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<b>TRANSMITTAL LETTER TO THE UNITED STATES          DESIGNATED/ELECTED OFFICE (DO/EO/US)          CONCERNING A FILING UNDER 35 U.S.C. 371</b>				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>10/0119992</b>	
INTERNATIONAL APPLICATION NO. <b>PCT/CA00/00790</b>		INTERNATIONAL FILING DATE <b>6 July 2000</b>		PRIORITY DATE CLAIMED <b>6 July 1999</b>	
TITLE OF INVENTION <b>METHODS AND COMPOSITIONS FOR REGULATING GUT MOTILITY AND FOOD INTAKE</b>					
APPLICANT(S) FOR DO/EO/US <b>Krantis and Durst</b>					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.					
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).					
<b>Items 11 to 20 below concern document(s) or information included:</b>					
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. (with references) 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment. 14. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4) 20. <input checked="" type="checkbox"/> Other items or information: Note: IPER has not been issued by the IPEA /EP as yet. No amendments to the application were made during preliminary examination.					



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**METHODS AND COMPOSITIONS FOR REGULATING  
GUT MOTILITY AND FOOD INTAKE**

15 This application claims the benefit of U.S. Provisional Application No. 60/143,054,  
filed July 6, 1999.

**Field of the Invention**

This invention is generally in the field of treating obesity and regulating food intake. In particular, this invention relates to compositions and methods of regulating food intake in which trichothecenes, derivatives and analogs thereof, or purinergic compounds are administered to alter gut motility and thereby satiety. The invention also relates to methods of screening for derivatives or analogs of trichothecenes and also for agonists and antagonists of purinergic receptors that are useful for regulating food intake.

25 **Background of the Invention**

Overeating leading to obesity is a major health problem. Obesity increases the risk of diabetes, heart disease, cancer and other chronic diseases, in addition to increasing the physical or mechanical restrictions imposed on the body. Although such adverse health effects of obesity are scientifically well documented and generally well understood by the public, the effective control of appetite and overeating on an individual basis has been a goal difficult to attain for millions of people. Approximately 25 percent of the children in North America are considered overweight or obese. North Americans alone spend approximately \$40 billion per annum on weight loss treatments, and this amount appears to be increasing. A recent study conservatively estimated the annual cost of treating obesity in Canada at \$1.8

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billion, representing 2.4 percent of the total health care expenditures for all diseases (see, "Cost of Obesity: \$1.8 billion," in *Pharmaceutical Manufacturers Association of Canada*, March 1999, page 11).

Currently available anti-obesity drugs work for the most part by targeting central nervous system (CNS) pathways to induce appetite suppression. However, such drugs have a number of CNS-related side effects, such as anxiety, and there is the potential for chronic health problems such as hypertension, cardiovascular disease, and diabetes. Another current approach to treating obesity is to control appetite by using "bulk" products, which are ingested instead of normal food. Such bulk products have the problem of altering nutritional status in that the bulk product does not contain the necessary range of desirable nutrients. Moreover, the individual who ingests a bulk product may refuse to consume any food, even desirable nutrients.

Drugs that suppress appetite are among the least desirable means to treat obesity because weight is usually regained once administration of such drugs is halted. Furthermore, serious undesirable side effects, including increased risk of diseases such as primary pulmonary hypertension may limit the use of such drugs. For example, the appetite suppressants fenfluramine and dexfenfluramine were recently pulled off the market by their manufacturers because of a potential for serious adverse effects on the lungs and heart.

Another type of obesity treatment that has emerged recently is the use of drugs that interfere with fat absorption from the small intestine. Such a drug may, for example, inhibit pancreatic enzymes used for fat digestion. Undigested fat is then passed through the intestines and excreted. Decreasing fat absorption can result in oily stool, oily spotting of undergarments, intestinal gas, frequent bowel movements, and decrease absorption of fat-soluble nutrients such as vitamins A, D, and E.

There is currently no medical approach that cuts weight gain without unhealthy side effects or increased risk of disease. Needs remain for effective treatments for obesity and methods of controlling weight gain in humans and other animals without untoward nutritional and medical side effects.

#### Summary of the Invention

This invention provides methods of treating obesity and controlling food intake in humans and other animals. The invention is based on the discovery of how mycotoxin trichothecenes produce food or feed refusal in humans and other vertebrate animals and also

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on the elucidation of the neural circuitry regulating patterns of gut motor activity ("gut motility"), which propels food through the organs of the gut. The methods of treatment described herein involve administering a compound that affects the pattern of gut motility, that is, the pattern of contractions, relaxations, and quiescence of the smooth muscle tissue of the organs of the gut. Stimulating the "fed pattern" of gut motor activity signals satiety, that is, a feeling of fullness, which shortens the time an individual spends eating or feeding. Thus, compounds that stimulate the fed pattern of gut motility are useful in methods of treatment where the goal is to limit food intake, as in treating obesity. Compounds that stimulate the "fasting pattern" or prolong or prevent the onset of the fed pattern of gut motility will tend to increase eating or feeding time because satiety is not signaled to the body. Such compounds are particularly useful in methods of increasing weight gain in animals, such as livestock and poultry raised as commercial sources of meat.

Methods of treating obesity provided by the invention comprise administering an effective amount of a trichothecene mycotoxin, or derivative thereof, which stimulates the fed pattern of gut motility. In a preferred embodiment of the invention, the methods of treating obesity comprise administering to an individual a trichothecene from the nivalenol-related group of structurally related compounds consisting of nivalenol; 4-deoxynivalenol ("DON",  $C_{15}H_{20}O_6$ ); trichothecol; trichothecin; 3-acetyldeoxynivalenol ("3-acetyl DON",  $C_{17}H_{22}O_7$ ); 7-acetyldeoxynivalenol; 3,15-diacetyldeoxynivalenol; 4-acetylnivalenol (fusarenon-X); 4,15-diacetylnivalenol. Other DON-based derivatives are also useful in the preferred methods of the invention, including DON carbonate (i.e., 3-hydroxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate,  $C_{16}H_{18}O_7$ ); 3-acetyl-DON carbonate (i.e., 3-acetoxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate,  $C_{18}H_{20}O_8$ ); 3-acetyl-DON benzylidene acetal (i.e., 3-acetoxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one,  $C_{24}H_{26}O_8$ ); DON-benzylidene acetal (i.e., 3-hydroxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one,  $C_{22}H_{24}O_7$ ); isopropylidene DON (i.e., 3-hydroxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one,  $C_{18}H_{24}O_6$ ); and isopropylidene-3-acetyl-DON (i.e., 3-acetoxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one,  $C_{20}H_{26}O_7$ ). More preferably, the methods of treating obesity comprise administering a trichothecene, such as DON or a DON-based derivative, to an individual at a dose which is non-toxic and non-emetic, but which stimulates the fed pattern of gut motility in the individual. The trichothecene, or derivative thereof, may be administered by any of a variety of routes, including orally or parenterally.

Alternatively, methods of treating obesity comprise administering a trichothecene analog, which is a compound that functions like a trichothecene to stimulate the fed pattern of gut motility. Trichothecene analogs may be structurally related to or structurally distinct from trichothecenes. Thus, trichothecene analogs may be derived from a trichothecene such as DON or may be any of a variety of compounds, including inorganic compounds, organic compounds, amino acids, peptides, polypeptides, proteins, nucleotides, nucleic acids, carbohydrates, lipids, and combinations thereof, which have the ability to stimulate fed pattern gut motility.

In another embodiment, the invention provides compositions and methods for regulating gut motility and treating obesity in an individual. Such methods of the invention comprise administering to the individual a compound that binds to and stimulates  $P_{2X1}$  purine receptors (purinoceptors), which are present in smooth muscle of gut tissues and are directly involved in regulating the fed pattern of gut motility. In particular, an agonist of the  $P_{2X1}$  purine receptor is a purinergic compound that binds the receptor to stimulate the fed pattern of gut motility. As with trichothecenes, stimulating the fed pattern of the gut motor activity with a purinergic compound signals satiety and thereby shortens feeding time and decreases food intake. Preferably, the agonist of the  $P_{2X1}$  receptor that is useful in treating obesity according to the invention is a "non-desensitizing" agonist of the purine receptor, in that molecules of the agonist are able to bind the  $P_{2X1}$  receptor and to stimulate the  $P_{2X1}$  mediated fed pattern of gut motility, without eventually blocking or inactivating the receptor. In a more preferred embodiment, the non-desensitizing agonist of the  $P_{2X1}$  receptor is a structural analog of ATP or of 2',3'-O-(2,4,6-trinitrophenyl)-ATP ("TNP-ATP") for use in methods of stimulating the fed pattern of gut motility and for treating obesity.

In another embodiment, the invention provides compositions and methods for increasing weight in an individual. Such methods comprise administering to the individual a desensitizing agonist or an antagonist, such as TNP-ATP, of the  $P_{2X1}$  receptor. A desensitizing agonist or an antagonist compound useful in such methods of the invention binds and blocks the  $P_{2X1}$  receptor, and thereby inhibits or prevents the fed pattern of gut motility and/or prolongs the fasting pattern of gut motility, which in turn increases feeding time and food intake. Such methods are particularly useful in raising commercial livestock and poultry for market.

In yet another aspect of the invention, methods are provided for identifying a compound that stimulates or inhibits (prevents) the fed pattern of gut motility by directly

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recording the pattern of gut motility *in vivo* in a test subject during administration and metabolism of the compound in the subject. Such methods may be used to test the regulatory activity of a compound, such as a known or new trichothecene compound, a trichothecene derivative compound, a trichothecene analog compound, an agonist of the  $P_{2X1}$  receptor, or an antagonist of the  $P_{2X1}$  receptor. The ability of a compound to regulate (stimulate or inhibit) patterns of gut motility can be measured using an *in vitro* gut bath assay, an *ex vivo* gut organ assay, or an *in vivo* assay. Trichothecene and purinergic compounds, and derivatives or analogs thereof, that are identified by such screening methods as capable of stimulating the fed pattern of gut motility may be used to treat obesity according to the methods of the invention, whereas compounds that inhibit fed pattern of gut motility may be used to increase food uptake, as in promoting weight gain in livestock and poultry for commercial markets.

#### Brief Description of the Drawings

Figure 1 shows a schematic diagram of the *in vivo* set up of Krantis et al. (*Can. J. Physiol. Pharmacol.*, 74: 894-903 (1996)), employed for recording gastrointestinal motility in anaesthetized experimental animals, such as a rat (1). In a detail (A) magnification of the rat gut, foil strain gauges (2) are shown attached (e.g., with a glue) to selected sites of gut organs, for example, the serosal surface of the gastric antrum (3), proximal duodenum (4), or distal ileum (5), along the longitudinal muscle layer. Wire leads are attached to an IBM computer data acquisition system (6).

Figure 2 shows a schematic representation of the neural pathways controlling fed and fasting patterns of gut motility in the duodenum and ileum. The arrangement of cholinergic (ACh), nitrgergic (NO), and purinergic (ATP) neurons is shown together with the different receptor targets and/or inputs: mus. (cholinergic muscarinic), 5-HT<sub>3</sub> (serotonergic), nic. (cholinergic nicotinic),  $P_{2X}$  (purinergic). A plus sign ("+") indicates a stimulatory input between neurons, and stimulation and contraction at smooth muscle of the gut; a minus sign ("-") indicates an inhibitory input. DON = deoxynivalenol, stimulator of gut hyperactivity (fed pattern) and satiety. NO = nitric oxide, which is a non-adrenergic, non-cholinergic (NANC) inhibitory transmitter in the proximal duodenum and which is also an inhibitory transmitter of the propagatory  $P_{2X}$ -purinergic and cholinergic (muscarinic, mus.) motor activity in duodenum and ileum. A circuit pathway of a nitrgergic interneurone (NO) with cholinergic nicotinic input (nic.) (far right) is not present in the ileum. ATP = adenosine triphosphate, de-sensitizing agonist of purinergic receptors, such as,  $P_{2X}$  receptors. ACh =

- acetyl choline, the cholinergic chemical signal that binds at muscarinic (mus.) receptors to excite motor neurons. 5-HT = 5-hydroxytryptamine (serotonin), binds to 5-HT<sub>3</sub> (serotonergic) receptors on neurons and is the major transmitter of enteric interneurons mediating neurogenic stimulation of NANC relaxations and cholinergic contractions of the smooth muscle of the gut. nic. = cholinergic nicotinic receptor of neurons.

- Figures 3A and 3B show diagrammatic chemical structures of 4-deoxynivalenol (DON) and related derivative compounds. Figure 3A shows diagrammatic chemical structures for DON (C<sub>15</sub>H<sub>26</sub>O<sub>6</sub>); 3-acetyl-DON (C<sub>17</sub>H<sub>22</sub>O<sub>7</sub>); isopropylidene DON (C<sub>18</sub>H<sub>24</sub>O<sub>6</sub>, designated EN139491); and isopropylidene-3-acetyl-DON (C<sub>20</sub>H<sub>26</sub>O<sub>7</sub>, designated EN139492).
- Figure 3B shows diagrammatic chemical structures for DON carbonate (C<sub>16</sub>H<sub>18</sub>O<sub>7</sub>, designated EN139494); 3-acetyl-DON carbonate (C<sub>18</sub>H<sub>20</sub>O<sub>8</sub>, designated EN139495); 3-acetyl-DON benzylidene acetal (C<sub>24</sub>H<sub>26</sub>O<sub>8</sub>, designated EN139496); and DON-benzylidene acetal (C<sub>22</sub>H<sub>24</sub>O<sub>7</sub>, designated EN139497). "Ph" represents a phenyl group; "OAc" represents an acetyl group.

- Figure 4 shows a recording of the spontaneous motor activity of the rat gastric antrum in a control animal showing the oscillatory appearance of contractile and relaxant responses. Vertical marks indicate time (t) at 0 and 50 minutes after start of recording. Administration of DON (first arrow to right of t = 0 minutes) at 10 mg/kg of body weight, i.v., abruptly attenuated the motor activity of gastric antrum. Within 40 minutes, the control motor pattern recovered, however, a proximal readministration of DON (second arrow) was without effect.

- Figure 5 shows an example of a recording of the *in vivo* motility pattern of the rat duodenum control activity. The spontaneous *in vivo* motility pattern of the duodenum control activity (no DON) consists of periodic "grouped" (G) and "intergroup" (I) activity. Vertical marks indicate time (t) at 0, 30, 120, and 150 minutes after start of recording. The first arrow after t = 30 minutes, indicates systemically administered DON (arrow) at 10 mg/kg of body weight (i.v.), which induced a sustained hyperactivity (46 ± 15 min). Following recovery of motor activity to control level, readministration of DON (arrow after t = 150 minutes) was without effect.

- Figures 6A-6D show data from a quantitative analysis of the effects of L-NAME and α, β-methylene ATP on frequency (Freq) and amplitude (Amp) of DON-induced relaxations in the rat duodenum (Figure 6A, frequency, and Figure 6B, amplitude) and ileum (Figure 6C, frequency, and Figure 6D, amplitude). Grouped motor activity in the presence of L-NAME and DON (broadly spaced diagonal bars) was equivalent to that with DON alone (closely



spaced diagonal bars).  $\alpha$ ,  $\beta$ -methylene ATP significantly attenuated the frequency and amplitude of DON induced relaxations (filled bars) to the level of control intergroup activity (no DON, open bars), in the duodenum (n = 8) and ileum (n = 4).

- Figures 7A-7D show results of a quantitative analysis of the effects of the 5-HT<sub>3</sub> receptor antagonist, granisetron, on spontaneous and DON-induced activity in the rat duodenum. Granisetron (i.v. or i.a., broadly spaced diagonal bars) selectively attenuated the frequency (Freq) and amplitude (Amp) of "grouped" relaxations (n = 6) (Figure 7A, frequency, and Figure 7B, amplitude) and contractions (n = 3) (Figure 7C, frequency, and Figure 7D, amplitude), however, it did not alter the stereotypic DON induced hyperactivity (compare closely spaced diagonal bars (DON alone) with filled bars (DON + granisetron)). Control "grouped" activity (open bars).

- Figure 8 is a bar graph showing the effects (as percent of control) of  $\alpha$ , $\beta$ -methylene ATP on DON-enhanced motor activity for contractions and relaxations of the duodenum in piglets. "Control" represents a group of piglets that received no DON and no  $\alpha$ , $\beta$ -methylene ATP. "DON" represents a group of piglets that received DON only (1 mg·kg<sup>-1</sup>). " $\alpha$ , $\beta$ -methylene ATP + DON" represents a group of piglets that received intra-arterial injection of  $\alpha$ , $\beta$ -methylene ATP (300  $\mu$ g/kg, i.a.) during DON (1 mg/kg) enhanced motor activity of the duodenum. Control group values were set as 100%. All other values are percent of control values. Open bars represent average (4 piglets) amplitude of relaxations. Filled bars represent average (5 piglets) frequency of relaxations. Open cross-hatched bars represent average (3 piglets) amplitude of contractions. Closely spaced cross-hatched bars represent average (2 piglets) frequency of contractions. " $\psi$ " indicates  $p < 0.05$  compared to control. " $\phi$ " indicates  $p < 0.05$  compared to DON enhanced activity.

- Figure 9 is a bar graph showing the effects (as percent of control) of  $\alpha$ , $\beta$ -methylene ATP on DON-enhanced motor activity (contractions and relaxations) of the ileum in piglets. "Control" represents the group of piglets that received no DON and no  $\alpha$ , $\beta$ -methylene ATP. "DON" represents the group of piglets that received DON only (10 mg/kg). " $\alpha$ , $\beta$ -methylene ATP + DON" represents the group of piglets that received intra-arterial injection of  $\alpha$ , $\beta$ -methylene ATP (300  $\mu$ g/kg, i.a.) during DON (10 mg/kg) enhanced motor activity of the ileum. Control group values were set as 100%. All other values are per cent of control values. Open bars represent average (4 piglets) amplitude of relaxations. Filled bars represent average (5 piglets) frequency of relaxations. Open cross-hatched bars represent

average (3 piglets) amplitude of contractions. Closely spaced cross-hatched bars represent average (2 piglets) frequency of contractions. " $\psi$ " indicates  $p < 0.05$  compared to control. " $\phi$ " indicates  $p < 0.05$  compared to DON enhanced activity.

Figure 10 shows a schematic representation of the arrangement of cholinergic, nitrenergic, GABAergic, purinergic and VIPergic neural elements within the proposed tonic and modulatory pathways controlling spontaneous motor activity in the rat duodenum and ileum. A circuit pathway having GABAergic and nitrenergic interneurons (NO) with cholinergic nicotinic input (nic.) (far right) is not present in the ileum. VIP = vasoactive intestinal peptide, which is an activator of nitrenergic prejunctional inhibition of motor innervations.

Figure 11 shows an example of a recording of the control spontaneous motor activity of the rat gastric antrum (site S1) and proximal duodenum (site D1). DON (10mg/kg of body weight ("bw"), administered i.v.) abruptly attenuated the antral motor activity, and induced a sustained hyperactivity in the duodenum (D1). Within 60 minutes, the control motor pattern recovered.

Figure 12 shows effects of 3-acetyl DON on rat gastrointestinal motor activity *in vivo*. Typical fasting pattern of gut motor activity in the duodenum (recorded at duodenal site D2) and the gastric antrum (S1) are shown prior to administration of 3-acetyl DON treatment. Following injection (vertical arrow) of 3-acetyl DON (10mg/kg, i.v.) the motor activity changed into a typical fed pattern motor activity lasting approximately 40 minutes. "MMC" is the "grouped" activity portion of fasting pattern of gut motility.

Figure 13 shows the effects of intravenously administered 3-acetyl DON at 10 mg/kg of body weight on spontaneous motor activity in the rat gastric antrum (S1) and duodenum (D2). Within 60 minutes, the control motor pattern recovered (see recording after 130 min). "MMC" is the "grouped" activity portion of the fasting pattern of gut motility.

Figure 14 shows typical *in vivo* recordings of the motor activity in the rat duodenum (D1) and gastric antrum (S1) illustrating the action of the compound EN139491 (10mg/kg bw, i.v.) on "fasting pattern" motor activity. The top panel shows 20 minutes of normal fasting pattern motor activity prior to administration of compound. Recording during this period shows that the duodenum displayed a typical pattern of low frequency spontaneous motor activity together with propagating "grouped" motor activity ("MMCs") and that the gastric antrum displayed a typically rhythmic motor activity. The second panel of recording shows the time of injection for EN139491 and development within 30 seconds of injection of a long lasting (40-60 minutes) hyperactivity in the duodenum and a simultaneous and parallel

attenuation of motor activity in the gastric antrum, characteristic of fed pattern motor activity. Recovery of fasting pattern motor activity is shown in the bottom panel as evidenced by periods of "grouped" MMC activity and less active "intergroup" activity.

Figure 15 shows the effect of compound EN139491 on duodenal motor activity recorded at site D2 (1.5 cm distal to the D1 strain gauge). Abbreviations are the same as in previous figures.

Figure 16 shows bar graphs of the effect on the amplitude of the relaxation component of gut motor activity by administration of the EN139491 DON derivative compound ( $10 \text{ mg} \cdot \text{kg}^{-1} \text{ i.v.}$ ) during spontaneous fasting pattern motor activity recorded *in vivo* from the proximal duodenum (D1) and gastric antrum of Halothane anaesthetized male Sprague Dawley rats. Amplitude of relaxation is expressed as percent of control "intergroup" activity. Amplitudes of the relaxation component of motor activity of "grouped" motor activity prior to administration of EN139491 (diagonal bar); for control "intergroup" motor activity prior to administration of EN139491 (open bar, set as 100%); and for hyperactivity after intravenous administration of EN139491 are shown. Each bar graph is the mean  $\pm$  SEM of data compiled from *in vivo* recordings obtained from 5-8 Sprague Dawley rats.

Figure 17 shows bar graphs as described in Figure 16, except that the effect of administration of EN139491 on gut motor activity on the frequency of the relaxation component of gut motor activity is shown.

Figure 18 shows bar graphs as described in Figure 16, except that the effect of administration of EN139491 on gut motor activity on the amplitude of the contraction component of gut motor activity is shown.

Figure 19 shows bar graphs as described in Figure 16, except that the effect of administration of EN139491 on gut motor activity on the frequency of the contraction component of gut motor activity is shown.

Figure 20 shows a typical *in vivo* recording of the motor activity in the rat duodenum (D1 and D2 duodenal recording sites) and gastric antrum (S1 antral recording site) illustrating the action of the DON derivative compound EN139492 (at  $10 \text{ mg} \cdot \text{kg}^{-1}$ , *i.v.*) on the fasting pattern of gut motor activity. The typical fasting pattern motor activity is evident in the recording prior to administration of EN139492. During this fasting pattern period, the duodenum (D1 and D2) displayed a typical pattern of low frequency spontaneous motor activity together with propagating "grouped" motor activity (MMCs), and the gastric antrum displayed a typically rhythmic motor activity. Within 30 seconds of administration of

EN139492 (vertical arrow) by injection, there developed a long lasting (40-60 minutes) hyperactivity in the duodenum and a simultaneous attenuation of motor activity in the gastric antrum, characteristic of the fed pattern of gut motor activity, though the effects on antral motor activity were not as sustained as in duodenal motor activity.

- 5 Figure 21 shows a typical *in vivo* recording showing the effects of intravenously injected  $P_{2X1}$  purinoceptor antagonist TNP-ATP ( $3.5 \text{ mg} \cdot \text{kg}^{-1}$ ) on spontaneous motor activity in the duodenum (at site D1) of a Halothane anaesthetized male Sprague Dawley rat. The recording shows that TNP-ATP did not evoke any response upon immediate injection (vertical arrow), but that, within 1 minute of injection, propagating motor activity (MMCs) was reduced. "Intergroup" motor activity was not significantly altered. Recovery of motor activity to within 90% of control level occurred within 20 minutes.
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- Figure 22 shows a typical *in vivo* recording showing the effects of intravenously injected TNP-ATP ( $3.5 \text{ mg/kg}$ ) on DON-induced ( $10 \text{ mg/kg}$  bw, i.v., vertical arrow above DON) fed pattern motor activity in the rat duodenum (site D1). TNP-ATP inhibitory effect on DON-induced fed pattern occurred within 1 minute of injection with TNP-ATP (vertical arrow above TNP-ATP).
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- Figure 23 shows the effects of intravenously administered TNP-ATP ( $3.5 \text{ mg/kg}$ ) on DON-induced fed pattern motor activity in the rat gastric antrum (S1) and duodenum (D2). Within 60 minutes, the control motor pattern recovered. The boxed portions of the recording show the initial actions of TNP-ATP at each site.
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- Figure 24 shows bar graphs of the amplitude of the relaxation component of gut motor activity recorded *in vivo* at the proximal duodenum (site D1) in Halothane-anaesthetized male Sprague Dawley rats. The amplitude of the relaxation component of DON-induced ( $10 \text{ mg/kg}$ , i.v.) gut hyperactivity in the absence of TNP-ATP (open bar) and presence of TNP-ATP ( $3.5 \text{ mg/kg}$ , i.v.) (checkered bar) is shown. The amplitude of the relaxation component of "grouped" MMC (diagonal bar) and the control "intergroup" activity (filled bar) is also shown. The amplitude of the relaxation component is expressed as the percent of the control "intergroup" relaxation amplitude, which was set at 100%. Asterisk indicates a significant ( $p < 0.05$ ) difference compared to control "intergroup" motor activity. Each bar graph is the mean  $\pm$  SEM of data compiled from *in vivo* recordings obtained from 5-8 Sprague Dawley rats. Each animal was its own control. D1 represents the recording obtained from a strain gauge positioned at 10 mm distal to the pyloric sphincter.
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Figure 25 shows bar graphs of the frequency of the relaxation component of gut motor activity recorded *in vivo* at the proximal duodenum (site D1) in Halothane-anaesthetized male Sprague Dawley rats. The frequency of the relaxation component of DON-induced (10 mg/kg, i.v.) hyperactivity in the absence of TNP-ATP (open bar) and presence of TNP-ATP (3.5 mg/kg, i.v.) (cross-hatched bar) is shown. The frequency of the relaxation component of "grouped" MMC (diagonal bar) and the control "intergroup" (filled bar) activity is also shown. The frequency of the relaxation component is expressed as the percent of the control "intergroup" relaxation frequency, which was set at 100%. Asterisk, statistics, and recording conditions of experiment as in Figure 24.

Figure 26 shows bar graphs of the amplitude of the contraction component of gut motor activity recorded *in vivo* at the proximal duodenum (site D1) in Halothane-anaesthetized male Sprague Dawley rats. The amplitude of the contraction component of DON-induced (10 mg/kg, i.v.) gut hyperactivity in the absence of TNP-ATP (open bar) and presence of TNP-ATP (3.5 mg/kg, i.v.) (cross-hatched bar) is shown. The amplitude of the contraction component of "grouped" MMC (diagonal bar) and the control "intergroup" activity (filled bar) is also shown. The amplitude of the contraction component is expressed as the percent of the control "intergroup" contraction amplitude, which was set at 100%. Asterisk, statistics, and recording conditions of experiment as in Figure 24.

Figure 27 shows bar graphs of the frequency of the contraction component of gut motor activity recorded *in vivo* at the proximal duodenum (site D1) in Halothane-anaesthetized male Sprague Dawley rats. The frequency of the contraction component of DON-induced (10 mg/kg, i.v.) hyperactivity in the absence of TNP-ATP (open bar) and presence of TNP-ATP (3.5 mg·kg<sup>-1</sup>, i.v.) (cross-hatched bar) is shown. The frequency of the contraction component of "grouped" MMC (diagonal bar) and the control "intergroup" (filled bar) activity is also shown. The frequency of the contraction component is expressed as the percent of the control "intergroup" contraction frequency, which was set at 100%. Asterisk, statistics, and recording conditions of experiment as in Figure 24.

#### Detailed Description of the Invention

This invention provides compositions and methods for treating obesity and regulating food intake by modulating the motor activity of gut organs in humans and other vertebrate animals. These methods are based on the discovery that trichothecene compounds, such as 4-deoxynivalenol (DON), stimulate the pattern of contractions and relaxations in organs of the

gut that normally occur when food is ingested. Stimulation of this "fed pattern" of gut motility signals satiety, that is, the feeling of fullness, which is an important factor that affects the time that an individual spends eating. Trichothecenes, such as DON, act at a site outside the organs of the gut and send a signal, which is sent down neural pathways leading to the smooth muscle of the gut organs. We have discovered that a specific receptor, the purinergic receptor  $P_{2X1}$ , present on cells of the smooth muscle of the small intestine is involved in regulating specific aspects of gut motor activity. Accordingly, compounds that act as agonists or antagonists of the  $P_{2X1}$  receptor are also useful in regulating gut motility and to controlling satiety and time spent eating.

In order to accurately describe the invention the following terms are defined:

As used herein, "gut" refers to the gastrointestinal tract consisting of the stomach, small intestine, and large intestine.

As used herein, "gut motility" or "gut motor activity" refers to the motor behavior of the smooth muscle in the gastrointestinal organs (stomach, small intestine, and large intestine) of humans and other animals which activity consists of periods of alternating muscular contractions and relaxations, as well as periods of quiescence or relatively little activity. For example, in normal, healthy humans and other animals, the frequency and amplitude of muscular contractions and relaxations of the small intestine become heightened when food is ingested in order to propel food aborally (forward) into the intestines for nutrient extraction and absorption (see, "fed pattern" of gut motility, below). Other patterns of gut motility may occur depending on the presence or absence of food in various parts of the gut organs. Furthermore, the proximal portion of a particular gut organ may exhibit motor behavior that differs from the activity in a distal portion of the organ, such as in the case of the duodenum (the beginning portion of the small intestine) and the ileum (the terminal portion of the small intestine).

As used herein, the "fed pattern", "fed pattern activity", and "segmentation" are synonymous and refer to the continuous pattern of contractions and relaxations of the small intestine of the gut in an animal, including humans, that normally occurs as the result of ingesting food. The fed pattern of gut motility propels ingested food through the gut for nutrient extraction and absorption, and eventually excretion of unabsorbed material as waste. The fed pattern of gut motor activity typically begins within minutes of ingesting food and is responsible for signaling satiety, that is, the feeling of fullness. Thus, satiety from the fed pattern of gut motility normally informs an individual that eating can be ended. Satiety is

sensed by an individual via the fed pattern of gut motility long before the brain has an opportunity to analyze the nutrient content of the blood (a separate process that takes place hours after food has been consumed and that is responsible for signaling cravings for specific nutrients, such as, proteins, carbohydrates, salt, and fats, which are maintained at specific levels for health).

The fed pattern is both characteristic and different for each organ and even different sites in the same organ of the gut. In the small intestine, the fed pattern is characterized by a continuous series of contractions and relaxations of the smooth muscle, which mixes intestinal contents, propels food aborally into the intestines, and delays antrograde propulsion to enhance substrate absorption (Lundgren et al., *Dig. Dis. Sci.*, 34: 264-283 (1989)). When measured and recorded *in vivo* by the method of Krantis et al. (*Can. J. Physiol. Pharmacol.*, 74: 894-903 (1996)), the fed pattern of gut motor activity in the duodenum is a characteristic intense pattern of hyperactivity whereas simultaneously in the gastric antrum, the fed pattern is characterized as a measurable suppression or decrease in recorded tissue motor activity. This fed pattern activity replaces the "fasting pattern" of gut motor activity (see, below), which occurs after food has been propelled through the gut for nutrient extraction. Fed pattern motility is activated primarily by peripheral autonomic ganglia via primarily vagal inputs and also, but to a lesser extent, is controlled by the central nervous system (CNS) (see, Yoshida et al., *J. Pharmacol. Exp. Therap.*, 256: 272-278 (1991); Tanaka et al., *J. Surg. Res.*, 53: 588-595 (1996); Chung et al., *Can. J. Physiol. Pharmacol.*, 70: 1148-1153 (1992)). Over-activation of autonomic nerves accelerates the onset and increases the duration of the fed pattern, concurrently increasing the frequency and amplitude of propagatory motor activity of the gut (Hall et al., *Am. J. Physiol.*, 250: G501-G510 (1986); Johnson et al., *Am. J. Surg.*, 167: 80-88 (1994), see also Examples below). As noted above, trichothecene mycotoxins, such as DON, can now be used in proper amounts to stimulate the fed pattern of gut motility.

"Fasting pattern" or "fasting cyclic motor pattern" of gut motor activity refers to the motor behavior of the gut in the absence of ingested food matter or before ingesting food, when no ingested material is present for propulsion from the stomach and into intestines. In the duodenum (the start of the small intestine), the fasting pattern of gut motor activity is characterized by alternating periods of spontaneous, irregular contractions and relaxations ("grouped" activity) and relatively quiescent periods ("intergroup" activity). An example of a duodenal fasting pattern with its alternating grouped and intergroup activities is shown in the

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early portion (between  $t = 0$  and  $t = 30$  minutes) of the recording of gut motility in Fig. 5. In the ileum (the end region of the small intestine), the fasting pattern is characterized by random contractile and/or relaxant motor activity or a generally quiescent state. Ingestion of food matter interrupts the fasting pattern of gut motility and stimulates the continuous activity of the fed pattern of gut motility.

Until recently, methods were not available for accurately measuring and characterizing gut motility in that only one component, either contraction or relaxation, could be measured under experimental conditions. More recently, however, Krantis and co-workers have developed a method of simultaneously measuring the contraction and relaxation components of gut motility for various organs of the gastrointestinal tract using miniaturized, flexible, foil strain gauges that can be attached *in vivo* to various locations on organs of the gut (see, Krantis et al., *Can. J. Physiol. Pharmacol.*, 74: 894-903 (1996)). In this method, wires from the gauges attached to the organs are connected to a computerized data analysis system (see, Fig. 1). The method of Krantis et al. (1996) may be used for pharmacological, neurological, and physiological studies of the gut using *in vivo*, *ex vivo* (organs positioned out of the body cavity), and *in vitro* (excised tissue from gut organs) procedures (see, Examples, below). The ability to simultaneously record contractions and relaxations in gut organs and at multiple sites within an organ provides a more precise characterization of gut motility, including distinct patterns of gut motility, and the effect of food and various chemical compounds on such patterns.

In the fasted state, the gut exhibits a cyclic motor behavior known as "MMC", "migrating motor complex", or "migrating myoelectric complex". MMCs are associated with interdigestive propulsion of intestinal contents and involve sequential activation of excitatory and inhibitory neurons to propagate cycles of contractions and relaxations that originate in the stomach and terminate at the ileum. An MMC cycle consists of three distinct phases: phase I is a quiescent phase; phase II is a period of irregular spiking of activity, and phase III is a short period of rapid spike bursts of activity. MMCs provide a basic intrinsic motor pattern, which functions as a "housekeeper" of the small intestine. For example, the highly propulsive phase III motor activity of each MMC cycle sweeps the intestinal lumen, clearing it of remnants to prevent bacterial overgrowth, back flow, and the accumulation of intestinal secretions (Caenepeel et al., *Dig. Dis. Sci.*, 34: 1180-1184 (1989)). Using the method of Krantis et al. (1996), it is now clear that gut motility may comprise both contractions and relaxations of smooth muscle. In the absence of food, the "grouped" activity of the fasting



pattern of intestinal gut motility appears to correspond to the same type of motor activity classically ascribed to phase III of MMCs. The presence of food in the intestinal lumen induces a switch from the fasting pattern to the fed pattern of gut motor activity.

The method of Krantis et al. (1996) has also enabled the discovery of the mode of action of compounds called trichothecenes or trichothecene mycotoxins on gut motility. As shown in Examples 1 and 2 (*infra*), the trichothecene 4-deoxynivalenol (DON) acts at sites outside the gut to stimulate the fed pattern of gut motility, which characteristically occurs after ingesting food and which signals satiety, that is, the sensation of fullness. These findings provide the mechanism to explain to the well-documented anorectic or feed refusal behavior of humans and other animals that have ingested crops contaminated with fungal species that produce DON or other trichothecenes. This invention provides a method of treating obesity that takes advantage of the ability of trichothecene compounds to induce the fed pattern of gut motility and satiety. Methods of treating obesity described herein comprise administering a trichothecene or similar acting compound, which stimulates the fed pattern of gut motility and, thereby, satiety. Sensing fullness, the individual is thus given a signal to stop eating. When circulating levels of the administered compound decrease, satiety will decline and the individual may continue to eat or feed.

This invention also provides methods of regulating food intake by administering an agonist or antagonist of the  $P_{2X1}$  purine receptor (purinoceptor), which mediates grouped relaxations of gut tissue. According to the invention, a trichothecene, such as DON, and derivatives and analogs thereof, which stimulates the fed pattern of gut motility, actually acts at a site outside the gut. From that remote site of action, a signal travels down neural pathways to smooth muscle cells of the gut that express  $P_{2X1}$  purine receptors, which are involved in regulating the fed pattern of gut motility (see, Fig. 2). Thus, a compound that binds and affects the  $P_{2X1}$  purinoceptor is acting at the terminal portion of the neural pathway, whereas DON or other such trichothecenes act upstream. According to the invention, one group of compounds useful in the methods described herein consists of analogs of adenosine triphosphate (ATP) which may act as agonists or as antagonists of the  $P_{2X1}$  purinoceptor. As explained below, certain types of agonists of the  $P_{2X1}$  purinoceptor bind the receptor and stimulate the fed pattern of gut motility. Such agonists of the  $P_{2X1}$  purinoceptor may be used in lieu of trichothecenes in methods of treating obesity. An antagonist of the  $P_{2X1}$  purinoceptor is a compound that binds and blocks the receptor, thereby switching off or

attenuating the fed pattern. Such  $P_{2X1}$  receptor antagonists suppress or prevent fed pattern and satiety and, thus, may be used to prolong eating time and promote weight gain.

#### Trichothecenes Useful in the Invention

- 5 Historically, trichothecene compounds were identified as one of the toxic secondary metabolites produced by various fungi that can contaminate crops, hence the designation trichothecene mycotoxins. Animals, including humans, that ingest such contaminated crops may experience a variety of pathological symptoms of mycotoxicosis, such as vomiting, diarrhea, hemorrhagic lesions in internal organs, alimentary toxic aleukia (ATA),
- 10 agranulocytosis, aplastic anemia, necrotic angina, inflammation of mucous membranes, refusal to eat, convulsions, sepsis, and in some cases, death (see, for example, Ueno, "Trichothecene Mycotoxins: Mycology, Chemistry, and Toxicology," in *Advances in Nutritional Research* 1980, 3: 301-353 (1980)).

- As used herein, "trichothecene mycotoxin" or "trichothecene" refers to a member of a
- 15 group of sesquiterpenoid family of chemical compounds based on the non-olefinic parent or core compound trichothecane. All trichothecenes are modified sesquiterpenes, and contain an olefinic (double) bond (hence, trichothecene) between carbon atoms at positions 9 and 10 (C-9, C-10), and an epoxy ring formed between carbon atoms at positions 12 and 13 (C-12, C-13). Thus, trichothecenes are also characterized as 12,13-epoxytrichothecene compounds.
- 20 Ueno classified naturally-occurring trichothecene mycotoxins into four groups based on structural and also fungal characteristics (see, for example, Ueno, 1980, *supra*). According to this classification scheme, members of a group of trichothecenes represented by nivalenol are non-macrocylic compounds that have the carbon-8 (C-8) substituted with a ketone (oxo-) group. In addition to nivalenol, the group of "nivalenol-related" trichothecenes includes such
- 25 naturally-occurring trichothecene mycotoxins as 4-deoxynivalenol (DON), trichothecolon, trichothecin, 3-acetyldeoxynivalenol (3-acetyl-DON), 7-acetyldeoxynivalenol, 3,15-diacetyldeoxynivalenol, 4-acetylnivalenol (fusarenon-X), and 4,15-diacetylnivalenol. As used herein, "DON", "4-DON", "deoxynivalenol", "4-deoxynivalenol", and "vomitoxin" all refer to the same trichothecene compound having the chemical structure shown in Fig. 3.
- 30 Thus, nivalenol differs from DON in that nivalenol contains a hydroxyl group at C-4, whereas DON lacks the hydroxyl group ("4-deoxy") at position 4.

Although clearly capable of causing severe and widespread incidences of toxicosis when ingested in sufficiently high quantities, DON is nevertheless considered as one of the

least potent trichothecenes with respect to sub-lethal toxicosis (see, for example, Prelusky et al., *Arch. Environ. Contam. Toxicol.*, 22: 36-40 (1992); Friend et al., *Can. J. Anim. Sci.*, 66: 765-775 (1986); Ueno, in Developments in Food Science IV, Trichothecenes, chemical, biological, and toxicological aspects (Elsevier, Amsterdam, 1983), pp. 135-146).

- 5 DON is also non-mutagenic as determined using a hepatocyte-mediated mutation assay with V79 Chinese hamster lung cells (Rogers and Heroux-Metcalf, *Cancer Lett.*, 20: 29-35 (1983)) or a skin tumorigenesis Sencar mouse model (Lambert et al., *Food Chem. Toxicol.*, 33: 217-222 (1995)). The cellular toxicity is not mediated by alteration in deoxyribonucleic acid (DNA) synthesis or repair (Bradlaw et al., *Food Chem. Toxicol.*, 23: 1063-1067 (1985); Robbana-Barnat et al., *Toxicology*, 48: 155-166 (1988)).

- 10 DON appears to undergo no extensive liver metabolism and is readily and predominantly eliminated in the urine. The derivatives deoxynivalenol glucuronide and de-epoxide DON have also been found in urine, apparently as the result of metabolism by microbes in the gut of animals that have received DON (see, for example, Worrell et al., 15 *Xenobiotica*, 19: 25-32 (1989); Lake et al., *Food Chem. Toxicol.*, 25: 589-592 (1987)). Moreover, as we have discovered, DON and other trichothecenes operate by stimulating responses outside the gut which have a pronounced effect on the muscular activity of the gut. That is, DON does not act directly on the smooth muscle or other structures of gut tissues and involves no harmful effects on gut tissues to achieve its effects on gut motility. Accordingly, 20 DON and the other nivalenol-related trichothecene compounds are particularly well suited for use in the methods described herein for treating obesity. Trichothecenes that are not structurally related to DON and the other nivalenol-related compounds, may also be used in methods of treating obesity provided they also stimulate the fed pattern of gut motility and at doses that do not result in any of the undesirable or severe symptoms of clinical 25 mycotoxicosis.

- Trichothecenes and derivatives thereof useful in the invention may be produced biologically from fungal cultures or by chemical synthesis. A variety of soil fungi that have been found contaminating and growing on cereal grains and other crops produce trichothecenes as secondary metabolites. Such fungi include species of *Fusarium*, 30 *Tricothecium*, *Trichoderma*, *Myrothecium*, *Cylindrocarpon*, and *Stachybotrys* (see, Ueno, 1980). Methods of producing and purifying DON and acetyl esters of DON (such as 3-acetyl DON and 15-acetyl DON) from *Fusarium* cultures have been described (see, *Can. J. Microbiol.*, 29: 1171-1178 (1983); Miller and Blackwell, *Can. J. Bot.*, 64: 1-5 (1986);

Greenhalgh et al., *Proceedings of the 6th IUPAC International Symposium on Mycotoxins and Phycotoxins*: 137-152 (Steyn, P.S., ed.) (Elsevier Press, Amsterdam, 1986); Miller and Arnison (*Can. J. Plant Path.*, 8: 147-150 (1986)). Thus, various tricothecenes useful in the methods described herein may be produced and extracted from fungal cultures using standard culture and production techniques (see, for example, Ehrlich et al., *Biochim. Biophys. Acta*, 932: 206-213 (1987); Ueno, 1980 (*supra*) and references cited therein). As noted above, DON is an abundant, natural contaminant of corn and wheat. Thus, DON and other tricothecenes may also be isolated from contaminated crops. Alternatively, they can be isolated from the Brazilian shrubs *Baccharis magapotomica* and *cordifolia* (Kupchan et al., *J. Org. Chem.*, 42: 4221-4225 (1977)). In addition, the invention provides new derivatives of DON that can be synthesized from DON or 3-acetyl-DON as described in the Examples section below.

In addition, fungi that produce tricothecenes may also be used to modify pre-existing tricothecenes. Such bio-transformation of DON and its derivatives has been undertaken in a variety of laboratories employing bacteria (Shima et al., *Appl. Environ. Microbiol.*, 63: 3825-3830 (1997)) or strains of *Fusarium*. For example, *F. roseum* maintained in peptone-supplemented medium converts 3-acetyldeoxynivalenol to DON (Yoshizawa et al., *Appl. Microbiol.*, 29: 54-58 (1975)). *F. nivale* can acetylate DON at the carbon at position 3 to give 3-acetyl-DON. Furthermore, these strains can deacetylate 7,15-diacetyl-DON to give 7-acetyl-DON.

The chemistry of the tricothecenes is well known so that various tricothecene compounds may be synthesized by chemical or biochemical procedures. The tricothecenes are sesquiterpene alcohols or esters chemically related by the tetra-cyclic 12,13-epoxytricothec-9-ene skeleton (Williams, *Arch. Environ. Contam. Toxicol.*, 18: 374-387 (1989)). It has been reported that certain tricothecenes related to 4-DON can also be prepared using the tricothecene T-2 toxin (4 $\beta$ ,15-diacetoxy-3 $\alpha$ -hydroxy-8 $\alpha$ -[3-methylbutyryloxy]-12,13 epoxytricothec-9-ene) as starting material, since it is produced abundantly by *F. tricinctum* and is easy to modify at the C-3 and C-8 positions (Ehrlich et al., *Appl. Environ. Microbiol.*, 50: 914-919 (1985); Udell et al., *Z. Naturforsch.*, 44: 660-668 (1989)). Removal of the C-3 hydroxyl of T-2 involves initial conversion of T-2 to the 3-phenylthionocarbonate and then reduction of this intermediate with tri-n-butyltin hydride to give 3-deoxy-T-2. This approach has been used by others to prepare 3-deoxyanguidine and 4-deoxyverrucarol (Schuda et al., *J. Nat. Prod.*, 47: 514-519 (1984)).

It has also been shown that to generate the C-8 oxo-functionality (that is, trichothecenes related to DON), T-2 and deoxy-T-2 are oxidized with selenium dioxide (Bamburg et al., *Tetrahedron*, 24: 3329-3336 (1968)). Additional derivatives, such as THP-7-DON (tetrahydropyranyl-7-DON) and DIDON (3, 7-dideoxynivalenol), have been produced by introducing a C-8 ketone into the T-2 toxin (Bamburg et al., 1968). This is achieved by first preparing 3-THP-T-2 triol and 3-deoxy-T-2 triol and then oxidizing them with manganese dioxide ( $MnO_2$ ) (Warpehosi et al., *J. Org. Chem.*, 47: 2897-2900 (1982)). Oxidation is not possible for preparation of 7-DON from T-2 tetraol because of competing ring cleavage reactions and the low solubility of T-2 tetraol in methylene chloride, which is used as the reaction solvent. For preparation of THP-7-DON from THP-T-2 triol,  $MnO_2$  oxidation is the only possible method, since the acetic acid used as the solvent for selenium dioxide oxidation, would remove the tetrahydropyranyl group. The side products in the  $MnO_2$  oxidations are trichothecene, with 15-carboxaldehyde functionality.

#### 15 Identification of DON-Related Compounds

DON-related trichothecene compounds can be identified using mass spectra, NMR (nuclear magnetic resonance) spectroscopy, infra-red spectroscopy, anisaldehyde staining, and TLC (thin layer chromatography), to detect the presence of one or more various structural features of DON or other trichothecenes.

20 All DON-related trichothecenes should have NMR spectra showing the expected AB coupling pattern from the C-13a and C-13b protons, and a proton at 6.5 ppm for C-10 (Cole and Cox, *Handbook of Toxic Fungal Metabolites* (Academic Press, New York, 1981), pp. 152-263).

25 With anisaldehyde staining, the 8-oxo-substituted (keto) trichothecenes form a lemon-yellow adduct, whereas compounds lacking the keto group at position 8 form red or brown colored adduct.

In the infrared spectra, the carbonyl group at position 8 absorbs at  $1660 - 1680\text{ cm}^{-1}$ . This confirms that the trichothecene retains the alpha, beta-unsaturated ketone functionality.

30 The mass spectral data for the acetylated analogs of DON should show the parent ion and fragment ions anticipated by loss of acetyl or acetic acid during the process.

Each trichothecene used in the methods described herein is preferably purified until it migrates as a discrete spot on thin layer chromatography (TLC). Homogeneity can be further assessed by using high pressure liquid chromatography (HPLC) in which the particular

trichothecene should elute as a single peak. GC-MS (gas chromatography and mass spectroscopy) analysis has also been useful in assessing purity, for example, as in showing a single peak for each type of species of purified acetylated trichothecene (Cole and Cox, 1981, *supra*).

- 5 As discussed below, the invention also encompasses compounds in which one or more of the above-described structural features of DON, or another trichothecene, has been modified or even eliminated to make a derivative compound that may also be used in the compositions and methods of the invention.

10 Trichothecene Derivatives and Analogs

- DON may be used in the methods of the invention, such as treating obesity, but must be used at a dose that stimulates the fed pattern of gut motility without causing any of the other undesirable side effects, including emesis (vomiting), one of the clinical symptoms of mycotoxicosis. Although the pharmaceutically acceptable dose of a trichothecene such as
- 15 DON can be determined using standard methods, it would be desirable to produce a modification in a trichothecene chemical structure to yield a structurally-related compound, which is even more benign with respect to possible untoward side effects. Such a "benign trichothecene" (for example, a "benign DON") is a derivative of the original trichothecene and is expected to be comparable or more potent than the original trichothecene in
- 20 stimulating the fed pattern of gut motility, but with fewer or no unwanted side effects. Hence, a preferred derived trichothecene (e.g., DON derivative) will exhibit one or more improved properties and will be preferred over a known trichothecene, such as DON, in methods of treating obesity.

- For example, various structural features of DON provide sites on the compound that are
- 25 particularly attractive candidates for modification to form DON derivatives. Advantageously, DON is a relatively small molecule and has a limited number of sites available for modification to alter activity of this compound. Such sites include the unsaturated bond between C-9 and C-10, the presence of the 12,13 epoxy ring, the presence of hydroxyl or other groups on the structural nucleus of the trichothecene, and the occurrence of hydroxyl or
- 30 other substituents at C-3, C-4, and C-15 (see, Figure 3A). In addition, space-filling molecular models reveal several features of the trichothecene nucleus that provide additional information in considering which site(s) to modify in providing a useful derivative of DON. Oxygen substituents in the A-ring (C-8 keto and C-7 hydroxyl groups) make this side of the

molecule more hydrophilic than when no substituent is present or when, like in T-2 toxin, an isovaleroxy side chain is present. The presence of hydroxyl groups at appropriate positions on the nucleus modifies biological activity. For example, the difference between 4-deoxynivalenol (DON) and nivalenol is the presence of a hydroxyl group at C-4 in DON.

- 5 Studies of the relationship between trichothecene structure and the characteristic property of trichothecenes to inhibit protein synthesis have also revealed several interesting features that may be considered in making derivatives of DON or other trichothecenes that may be employed in the methods of the invention (see, Erlich et al., *Biochim. Biophys. Acta*, 923: 206-213 (1987); Rotter et al., *Env. Health*, 48: 1-34 (1996)). With respect to inhibition
- 10 of protein synthesis, the most potent trichothecenes lack substitution in the A-ring containing the 8-oxo substituent, or have esterified hydroxyls. When a C-7 hydroxyl is present, hydrogen (H) bonding to the C-8 keto can occur, but this makes the ring more sterically strained. H-bonding can also occur between the C-15 and C-7 hydroxyl groups. Removal of the C-7 hydroxyl group exposes the C-15 substituent on the side of the trichothecene away
- 15 from the 12,13-epoxide. In addition, the C-7 hydroxyl group must contribute to trichothecene potency since nivalenol, which possesses the C-7 hydroxyl group, is an order of magnitude more potent than 7-DON, which lacks this hydroxyl group. It is, thus, understood that comparable or corresponding sites in DON and other trichothecenes, especially the nivalenol-related trichothecenes, may be considered as potential sites for modification to
- 20 produce a derivative of DON or other trichothecene, which is useful in the compositions and methods of this invention.

- Examples of derivatives of the trichothecene DON useful in the compositions and methods of the invention include compounds referred herein as 3-acetyl-DON ( $C_{17}H_{22}O_7$ ); isopropylidene DON (3-hydroxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one,
- 25  $C_{18}H_{24}O_6$ , designated EN 139491); isopropylidene-3-acetyl-DON (3-acetoxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one,  $C_{20}H_{26}O_7$ , designated EN 139492); DON carbonate (3-hydroxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate,  $C_{16}H_{18}O_7$ , designated EN139494); 3-acetyl-DON carbonate (3-acetoxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate,  $C_{18}H_{20}O_8$ , designated EN139495); DON benzylidene acetal (3-hydroxy-7,15-
- 30 benzylidene-12,13-epoxy-9-tricothecin-8-one,  $C_{22}H_{24}O_7$ , designated EN139497); and 3-acetyl-DON benzylidene acetal (3-acetoxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one,  $C_{24}H_{26}O_8$ , designated EN139496) (see, Figures 3A and 3B). These compounds may also serve as "parent" compounds or starting materials that may be further modified to yield

additional new DON derivative compounds, which, preferably, exhibit one or more improved properties that will make the new derivative compound preferred over the parent compound or other known trichothecenes for use in compositions and methods for regulating gut motility described herein.

- 5 It is also understood that an alternative to DON or other trichothecene need not be a structurally related, derivative compound, as any compound that regulates gut motility at a dose having minimal or no untoward side effects may be useful in the methods described herein.

- According to the invention, a trichothecene analog is any compound that mimics one or more of the characteristic and desirable biochemical activities of a trichothecene, whether or not the compound has structural characteristics of a trichothecene. Like DON, trichothecene analogs useful in the methods of the invention regulate gut motility by acting outside the gut, in the periphery. In particular, trichothecene analogs which are useful in the methods described herein for treating obesity, act outside the gut to stimulate the fed pattern of gut motility and, thereby, signal satiety to stop eating. Such trichothecene analogs may be structurally related or chemically derived from DON or another trichothecene (see above); an inorganic molecule; an organic molecule unrelated to the trichothecenes; biomolecules, such as nucleotides, nucleic acids, peptides, polypeptides, proteins, carbohydrates, lipids; or combinations thereof. Whether a particular compound is a trichothecene analog according to the invention may be determined using one or more methods of screening for trichothecene activities described herein.
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#### Agonists and Antagonists of the $P_{2X1}$ Purine Receptor (Gut Neurotransmitter Receptor)

- Compounds that bind the  $P_{2X1}$  subtype of purinoceptor found in gut tissue may also be used in the methods described herein. The  $P_{2X1}$  purinoceptor is a neurotransmitter receptor present in smooth muscle of the gut and is involved in the control of gut motility (see, Examples, Fig. 2). Adenosine triphosphate (ATP) is a naturally occurring ligand of the  $P_{2X1}$  receptor. In the duodenum and ileum of the small intestine, stimulation of purinergic motor neurons releases ATP. ATP initially acts as an agonist to the  $P_{2X1}$  purinoceptor in that the first ATP molecule to bind to a  $P_{2X1}$  purinoceptor on a smooth muscle cell signals an inhibition of the smooth muscle, which then relaxes. As noted above, such relaxation is a component of gut motility that can be detected and measured using the method of Krantis et al. (1996). However, a molecule of ATP appears to remain bound to the  $P_{2X1}$  purinoceptor
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and thereby desensitizes the muscle to additional relaxation by ATP because other ATP molecules cannot bind the receptor to signal additional relaxation events (Smits et al., *Br. J. Pharmacol.*, 303: 695-703 (1996)). Accordingly, the  $P_{2X1}$  receptor mediated pathway for gut motor activity is blocked, resulting in an observable attenuation of all gut motor activity, which cannot be influenced by additional ATP (development of tachyphylaxis). ATP is, therefore, a "desensitizing" agonist, which prevents any further relaxations, which are critical in both the fed pattern and fasting pattern of gut motility. ATP is capable of binding to all types of purinergic receptors. The synthetic ATP analog,  $\alpha, \beta$ -methylene ATP, is also a desensitizing agonist, but is specific for  $P_{2X}$  species of purinoceptors. Furthermore, since the  $P_{2X1}$  subtype of purinoceptor is the  $P_{2X}$  species of receptor involved in the relaxation component of gut motility, studies using  $\alpha, \beta$ -methylene ATP provide data that accurately reveal the specific neurophysiological features of relaxation in gut motility (see, Examples).

In contrast to a desensitizing agonist of the  $P_{2X1}$  receptor, such as ATP or  $\alpha, \beta$ -methylene ATP, a "non-desensitizing" agonist of the  $P_{2X1}$  receptor has receptor binding properties that are necessary to provide continual stimulation of relaxations for gut motor activity. In particular, a non-desensitizing agonist of the  $P_{2X1}$  receptor is a compound that binds but does not block the receptor. Each molecule of a non-desensitizing agonist is able to bind the  $P_{2X1}$  receptor, evoke a relaxation, and then dissociate to be replaced by another of its kind, which in turn signals another relaxation, and so on. Thus, non-desensitizing agonists of the  $P_{2X1}$  receptor are able to stimulate relaxation events as long as molecules of the non-desensitizing agonist are available for binding to the  $P_{2X1}$  receptor. Non-desensitizing agonists of the  $P_{2X1}$  receptor stimulate the fed pattern of gut motility. Thus, non-desensitizing agonists of the  $P_{2X1}$  receptor are chemical alternatives to using DON, other trichothecenes, or trichothecene analogs in the methods of treating obesity described herein.

A desensitizing agonist (see, above) or an antagonist of the  $P_{2X1}$  receptor blocks the receptor and attenuates gut motility. Such compounds may be used to prevent or inhibit the fed pattern of gut motility and, thereby, inhibit satiety. Inhibiting satiety will promote longer eating or feeding time because the feeling of fullness is not evoked. According to the invention, an antagonist, such as 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate ("TNP-ATP"), or a desensitizing agonist, such as  $\alpha, \beta$ -methylene ATP, that acts at  $P_{2X1}$  receptors in gut tissue is useful in methods of prolonging eating time and increasing weight gain. Such a goal is particularly useful in the meat and poultry industry where increasing

weight of livestock or decreasing the time required to bring an animal to a marketable weight is commercially desirable and advantageous.

- A number of structural analogs of ATP and some of their pharmacological characteristics and receptor binding properties are known (see review by Harden et al., in *Annu. Rev. Pharmacol. Toxicol.*, 35: 541-579 (1995)). Such compounds may serve as candidate compounds that can be further screened for the ability to bind the  $P_{2X1}$  subtype purinoceptor and affect gut motility. Alternatively, the chemical structures of such known ATP analogs may be further modified to make other ATP analogs that can then be screened for the ability to act as non-desensitizing agonists, desensitizing agonists, or antagonists of the  $P_{2X1}$  receptor that can be used in the various methods described herein.

- The ATP analog TNP-ATP is a  $P_{2X}$  purinoceptor antagonist that has been used *in vitro* as a  $P_{2X}$  subtype selective antagonist (whole tissue  $IC_{50}$  in  $\mu M$  range) to determine the role of  $P_{2X1}$  and  $P_{2X3}$  homomeric and  $P_{2X2/3}$  heteromeric purinoceptors (see, Lewis et al., *Br. J. Pharmacol.*, 124: 1463-1466 (1998); Virginio et al., *Mol. Pharmacol.*, 53: 969-973 (1998)).  $P_{2X3}$  receptors are reported to be expressed only on sensory neurones (Evans et al., *Semin. Neurosci.*, 8: 217-223 (1996)). As shown herein, the TNP-ATP antagonist is useful to show the direct involvement of the  $P_{2X1}$  subtype purinoceptor in regulating the fed pattern of gut motility *in vivo*. Since TNP-ATP is able to prevent or inhibit the fed pattern of gut motility induced by DON, other trichothecene compounds, and derivatives thereof, this  $P_{2X1}$  antagonist may, itself, be used in methods of the invention for prolonging onset of satiety and for increasing food uptake. Such methods are particularly useful for preparing livestock and poultry for market. Furthermore, TNP-ATP may be used as a parent molecule for producing derivative compounds having enhanced or improved properties affecting gut motility.

- Another class of compounds that may serve as a source of agonists or antagonists of the  $P_{2X1}$  receptor useful in this invention are anthraquinone-sulfonic acid derivatives originally described by Bohme et al. (*Chromatogr.*, 69: 209-213 (1972)). Such derivatives may be viewed as ATP analogs and include a triazinyl moiety, which has been shown to antagonize certain ATP-mediated actions in the guinea pig (see, Kerr and Krantis, *Proc. Austr. Physiol. Soc.*, 10: 156P (1979)). It is expected that those anthraquinone-sulfonic acid derivatives that are capable of binding the  $P_{2X1}$  receptor and regulating (that is, either stimulating or inhibiting) patterns of gut motility may be useful in various methods described herein.

Another approach for developing non-desensitizing agonists of the  $P_{2X1}$  receptor that are useful in treating obesity is to develop compounds from sulfonyl ureas, for example, by

- replacing the triphosphate moiety of the parent compound (adenosine 5'-tetrahydrogen triphosphate) with unique, innovative acidic functionalities that are known to mimic the charge distribution in diphosphate or triphosphate, but that have never before been combined with the adenosine molecule. Importantly, the adenosine-SO<sub>2</sub>-NH-CO moiety is available for
- 5 combinatorial chemistry on a polymer base (Chiron Technologies). Compounds that bind the P<sub>2X1</sub> receptor and stimulate the fed pattern of gut motility are useful in methods of treating obesity according to the invention.

### Screening Methods

- 10 Various methods may be employed to identify those trichothecenes, trichothecene analogs, non-desensitizing P<sub>2X1</sub> receptor agonists, P<sub>2X1</sub> receptor antagonists, and other gut motility-regulating compounds that are useful in the methods and compositions of the invention.

- Specific antibodies for detecting DON and 15-acetyl DON have been made and used
- 15 in ELISAs (enzyme linked immunosorbent assays) (see, Sinha et al., *J. Agric. Food Chem.*, 43: 1740-1744 (1995)). Thus, antibodies to DON and other trichothecenes may be employed in various immunological procedures, such as ELISA, to rapidly screen for derivatives or related trichothecenes that may also be employed in the methods described herein.

- Structure-function relationships for a large number of 12,13-epoxytrichothecenes have
- 20 been determined using *in vitro* cell cultures of Vero (Green Monkey kidney) cells, murine erythroleukemia (MEL) cells, and rat spleen lymphocytes. For example, such cell culture systems were used to test the ability of various 12,13-epoxytrichothecenes to inhibit peptidyltransferase activity and, hence, protein synthesis (see, for example, Erlich and Daigle, *Biochim. Biophys. Acta*, 923: 206-213 (1987); Rotter et al., *J. Toxicol. Env. Health*, 48:1-34
- 25 (1996)). In particular, trichothecenes bind to the 60S subunit of the eukaryotic ribosome and, thereby, interfere with peptidyltransferase. The degree of structural substitution on the trichothecene sesquiterpene affects the binding characteristics to the peptidyltransferase and hence the degree of inhibition of this enzyme (Erlich et al., 1987; Rotter et al., 1996).

- Cell cultures as described above can be employed to test or screen compounds of
- 30 unknown activity as possible candidate compounds useful in the compositions and methods of treatment described herein. Such cell-based testing and screening methods are particularly useful to test and characterize various trichothecene or derivative compounds of unknown activity, such as newly synthesized or discovered compounds having structural features of

known trichothecenes, such as DON or other nivalenol-related trichothecenes. Other compounds that are not structurally related to known trichothecenes may also be screened using such cell cultures.

In cell-based screening methods, each test compound may be compared to one or more standard preparations of a known trichothecene, such as DON, which is typically prepared in stock solutions (10 µg/ml) in dimethyl sulfoxide. The concentration of dimethyl sulfoxide is adjusted so that it is always 1% (v/v) or less during incubations with the cells. Trichothecenes are generally stable for up to one year at room temperature (27°C).

Preferably, candidate compounds may also be screened and characterized using the method of Krantis et al. (1996), which uses miniaturized foil strain gauges and a computerized data analysis system to precisely and simultaneously record relaxations and contractions of smooth muscle in the gut. This is a means of screening compounds directly for their effects on gut motility. As noted above, the method of Krantis et al. (1996) is able to provide an actual recording of the effect of a compound on fed and fasting patterns of gut motility *in vivo*, *ex vivo*, or *in vitro* (see, Examples 1 and 2). Such methods of screening or identifying a new compound for use in the compositions and methods of the invention may also comprise comparing the effect that a candidate compound has on gut motor activity with the effect that a known trichothecene, such as DON, has on gut motor activity.

#### Binding Assays and Screens for P<sub>2X1</sub> Agonists or Antagonists

Candidate compounds (also referred to as "lead" or "test" compounds) can also be tested or screened for the ability to bind or block the P<sub>2X1</sub> subtype of purine receptor, which is the purinergic receptor expressed on smooth muscle and particularly involved in controlling the relaxation component of gut motility in the small intestine. Much is now known about the structure of P<sub>2</sub>-purinoceptors (see, for example, Virginio et al., *Mol. Pharmacol.*, 53:969-973 (1998); Humphrey et al., *Naunyn Schmiedeberg's Arch. Pharmacol.*, 352:585-596 (1995); Bo et al., *Br. J. Pharmacol.*, 112:1151-1159 (1994); and reviews in Burnstock, G., *Ciba Found. Symp.*, 198: 1-28 (1996) (P<sub>2</sub> receptor classification) and in Surprenant, A., *Ciba Found. Symp.*, 198: 208-219 (1996) (functional properties of native and cloned P<sub>2X</sub> receptors)). The ability of a compound to bind the P<sub>2X1</sub> purinoceptor may also be compared with that of a known receptor ligand, such as ATP or an ATP analog such as  $\alpha$ ,  $\beta$ -methylene ATP or TNP-ATP.

Successful radiolabeling of cell surface receptors for extracellular stimuli depends on the availability of ligands of high affinity, stability, and protein-binding specificity. Until recently, there were no selective antagonists for specific subtypes of  $P_2$  purinoceptors, and the several compounds that had been shown to competitively inhibit  $P_2$  purinoceptors (for example, suramin, reactive blue 2) did so with only micromolar affinity and lacked specificity in that they interacted with many other proteins. A consistent problem has been that binding assays have been carried out under conditions, for example, with membranes, which are very different from the conditions in which biological responses to the receptor can be measured. Accordingly, direct correlations between binding constants and receptor activity constants have been difficult to make. Agonists of  $P_2$ -purinoceptors also present problems, since their binding affinities are only slightly higher than their affinities for other ATP binding proteins, and they are subject to hydrolysis by nucleotide hydrolyzing enzymes.

[ $^3H$ ]-labeled  $\alpha,\beta$ -methylene ATP has been used as a radioligand for  $P_{2X}$ -purinoceptors in preparations of urinary bladder and vas deferens smooth muscle. Generally, agonist binding affinities follow those observed in intact tissues. For example, the differences in apparent binding affinity of  $\alpha,\beta$ -methylene ATP and 2-methyl-S-ATP for competition at the vas deferens binding site were only about 30-fold, and many nucleotides that are supposedly not  $P_{2X}$ -agonists also fully inhibit radioligand binding. The density of binding sites labeled with [ $^3H$ ]  $\alpha,\beta$ -methylene ATP far exceeded that observed with all other neurotransmitter receptors.

Enteric smooth muscle expressing  $P_{2X1}$  receptors can be dissociated, and the isolated smooth muscle cells maintained in primary culture. These cultures can be used in a binding assay for lead or candidate compounds that are able to act as agonists or antagonists of the  $P_{2X1}$  receptor. Alternatively, embryonic kidney 293 cells which express the  $P_{2X1}$  receptors may be used (Virginio et al, *Mol. Pharmacol.*, 53:969-973 (1998)). This receptor subtype is also expressed in platelets and megakaryoblastic cell lines (Vial et al., *Thromb. Haemost.*, 78: 1500-1504 (1997)), as well as in HL60 cells (Buell et al, *Blood*, 87: 2659-2664 (1996)). Accordingly, any cell, including recombinantly modified cells, that expresses the  $P_{2X1}$  receptors in culture may be useful in screens for agonists or antagonists of the  $P_{2X1}$  receptor.

The  $P_{2X1}$  purine receptor has been purified (Valera et al., *Nature*, 371: 516-519 (1994)) and cloned (Sun et al., *J. Biol. Chem.*, 273: 11544-11547 (1998)). The binding characteristics for recombinant  $P_{2X1}$  receptors have been described (Michel et al., *Br. J. Pharmacol.*, 118:1806-1812 (1996)). A purified  $P_{2X1}$  receptor may be attached by any of a

variety of linking agents to a solid substrate, such as the surface of a well in a microtiter plate, a resin particle, or the surface of an assay chip. Such arrangements allow very small quantities of compounds to be tested for the ability to bind to the receptor. Furthermore, the robotic technology that is available for screening samples in microtiter plates and assay chips permits hundreds or thousands of compounds to be accurately and continuously screened in hours with minimal supervision by the skilled practitioner.

Lead or candidate compounds identified as having an activity in one of the above screening methods can be further evaluated using an *in vitro* assay, *ex vivo* gut organ assay, and/or an *in vivo* assay for gut motility, for example, by the method of Krantis et al. (1996) (see, Example 1). In an *in vitro* gut organ bath assay, portions of a gut organ, for example, segments of the duodenum, jejunum, and ileum of the small intestine, are excised from an animal and placed in a physiological maintenance medium, such as Krebs solution at physiological body temperature. Individual gut segments are usually mounted to record circular muscle activity, preferably at two attachment points. A compound may be injected, mixed, or applied to the extricated gut organ segments, and the effect on the organ's motility measured. In an *ex vivo* gut organ assay, the gut organs of an anesthetized animal are exposed, but maintained intact and at physiological conditions. A test or lead compound may then be conveniently applied (topically) directly on the organ, and the effect on the organ's motility monitored. In an *in vivo* assay, a compound may be injected into or ingested by an animal, and the effect on gut motility measured directly.

Sources of compounds to be tested or screened for use in the compositions and methods described herein include, without limitation, small molecule collections, combinatorial libraries, growth media or cell extracts from fungal, bacterial, and various eukaryotic cell cultures or fermentations, and biological fluids, tissues, and serum samples from humans and other animals.

#### Methods of Treatment, Pharmaceutical Compositions, Modes of Administration

The invention provides pharmaceutical compositions, which are used in methods of treating obesity. Other compositions of this invention are formulated for administering to animals to promote weight gain, which is especially useful in raising commercial livestock and poultry for market. Humans and other vertebrate animals have the same basic gut neurophysiology with respect to controlling gut motility. Accordingly, animals that can be treated using the methods described herein include, without limitation, humans and other

primates, swine, cattle, sheep, birds (poultry and other birds), horses, cats, dogs, and rodents, including hamsters, guinea pigs, rats, and mice. Both pharmaceutical compositions and compositions for administration to other animals described herein contain an effective amount of a compound to achieve the desired effect on gut motility without significant or undesirable side effects.

According to the invention, obesity is treated by administering to a human or other animal an effective amount of DON or other trichothecene, trichothecene derivative, trichothecene analog, or non-desensitizing agonist of the  $P_{2X1}$  purinoceptor to stimulate or activate the fed pattern of gut motility and, thereby, signal satiety. In contrast, a  $P_{2X1}$  purinoceptor antagonist, such as TNP-ATP, or a desensitizing agonist, such as  $\alpha, \beta$ -methylene ATP, may be administered to a human or other animal to prevent or inhibit the fed pattern of gut motility and, thereby, prolong eating time and promote weight gain.

Such compositions may be in any of a variety of forms particularly suited for the intended mode of administration, including solid, semi-solid or liquid dosage forms, for example, tablets, lozenges, pills, capsules, powders, suppositories, liquids, powders, aqueous or oily suspensions, syrups, elixirs, and aqueous solutions. Preferably, the pharmaceutical composition is in a unit dosage form suitable for single administration of a precise dosage, which may be a fraction or multiple of a dose which is calculated to produce the desired affect on gut motility. The compositions will include, as noted above, an effective amount of the selected compound in combination with a pharmaceutically acceptable carrier and/or buffer, and, in addition, may include other medicinal agents or pharmaceutical agents, carriers, diluents, fillers and formulation adjuvants, or combinations thereof, which are nontoxic, inert, and pharmaceutically acceptable. In liquid mixtures or preparations, a pharmaceutically acceptable buffer, such as a phosphate buffered saline may be used. By "pharmaceutically acceptable" is meant a material that is not biologically, chemically, or in any other way, incompatible with body chemistry and metabolism and also does not adversely affect any other component that may be present in the pharmaceutical composition.

For solid compositions, conventional nontoxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate, and the like. Pharmaceutically acceptable liquid compositions can, for example, be prepared by dissolving or dispersing an active compound that regulates gut motility as described herein and optimal pharmaceutical adjuvants in an excipient, such as, water, saline, aqueous dextrose, glycerol, ethanol, and the

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like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, for example, sodium acetate, triethanolamine oleate.

- 5           Standard methods of preparing dosage forms are known, or will be apparent, to those skilled in this art (see, for example, Remington's Pharmaceutical Sciences (Martin, E.W. (ed.) latest edition Mack Publishing Co., Easton, PA). In the case of DON and other trichothecenes, a dose is prepared that does not result in emesis (vomiting). Such sub-emetic doses are readily determinable as has been demonstrated in animal studies (see, Examples 1  
10 and 2).

- The primary active ingredient of a composition of this invention is a compound which is a trichothecene, trichothecene analog, an agonist of the  $P_{2x1}$  receptor, or an antagonist of the  $P_{2x1}$  receptor that affects (modulates) gut motility. Trichothecenes such as DON are clearly capable of exerting their activity on gut motility when ingested. Accordingly, a  
15 preferred composition of this invention is formulated for oral administration. Such compounds may also be administered parenterally, for example, by intravenous, intramuscular, or intraperitoneal injection.

- For oral administration, which is preferred, compositions of the invention may be formulated as fine powders or granules containing of the compound that affects gut motility  
20 and may also contain diluting, dispersing, and/or surface active agents. Compositions for oral administration may also be presented in water or in a syrup as a solution or suspension, in pills, tablets, capsules or sachets in the dry state, or in a nonaqueous solution or suspension wherein suspending agents may be included. Binders and lubricants may also be used in compositions for oral administration. Where desirable or necessary, flavoring, preserving,  
25 suspending, thickening, or emulsifying agents may be included. Tablets and granules are preferred oral administration forms, and these may be coated.

- Parenteral administration, if used, is generally a method of injection. Injectable preparations can be prepared in conventional forms, either liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.  
30 For most purposes, a compound useful in regulating gut motility may be injected intravenously in a pharmaceutically acceptable buffer. However, it is within the scope of this invention that such a compound may alternatively be prepared as a bolus, which may contain a mordant for gradual release from an injection site. One approach for parenteral



administration involves use of a slow release or sustained release system, such that a constant level of dosage is maintained (see, for example, U.S. Patent No. 3,710,795).

The exact, effective amount of a compound useful in regulating gut motility in the compositions and methods described herein will vary from subject to subject, depending on the age, weight and general condition of the subject, the degree of obesity being treated, the particular compound used, its mode of administration, and the like. Thus, it is not possible to specify an exact amount for an ideal dose applicable to all individuals. However, it is expected that generally a trichothecene such as DON will be used or tested in a range of 0.01 – 100 mg/kg body weight. Furthermore, the useful dosage selected for a particular individual will be a sub-emetic dose, that is, a dose that does not evoke vomiting in that individual. For commercial pharmaceutical compositions, it is understood that a pharmaceutically effective and suitable amount of trichothecene, trichothecene derivative, trichothecene analog, P<sub>2X1</sub> receptor agonist, or P<sub>2X1</sub> receptor antagonist will be determined, in the case of human use, by the healthcare professional in studies acceptable to the standards of the United States Food and Drug Administration (or comparable agency). For use in animals, an appropriate composition will be determined and formulated according to the standards and practices for commercial livestock feed or veterinary medicine.

Additional embodiments and features of the invention will be apparent from the following non-limiting examples.

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#### Examples

##### Example 1

The following example shows that DON acts at sites outside the gut and interferes with specific intrinsic neural pathways of the stomach and small intestine, giving rise to altered patterns of motor activity. These findings show that DON induces loss of appetite (as illustrated by feed refusal in animals) and support a method of inducing such loss of appetite.

Normal gastrointestinal motility is dependent on the intrinsic (enteric) neural networks of the gut wall, with modulatory inputs from the periphery and central nervous system (CNS). The intrinsic circuitry coordinates reflex activity, such as the peristaltic reflex, or complex patterned motor activity, such as interdigestive migrating myoelectric complexes (MMCs) that occur within the fasting pattern and segmentation driving fed pattern.

The effects of DON on the pathways controlling interdigestive spontaneous motor activity of the rat stomach and intestine were examined. Even though rats do not possess an emetic reflex, they do exhibit the sickness and discomfort associated with vomiting (Andrews, *Br. J. Anaesth.*, 69: 2s-19s (1992), Rapley, W.A. and Hirst, M., Abstract in *Lab. Anim. Sci.*, 38: 504 (1988)). The effects of low-threshold levels of DON on the interdigestive motor pattern *in vivo* were examined. Additional information was obtained from *in vitro* gut bath experiments to obtain adjunct pharmacological information.

Experiments were performed on male Sprague-Dawley rats (Charles River) weighing 250-350 grams. All experimental protocols were carried out according to the guidelines of the Animal Care Committee at the University of Ottawa.

Individual rats, fasted for 24 hours with free access to water, were anesthetized using a Halothane (4%) in oxygen mixture. Rats were maintained under Halothane (2%) anesthesia on a heated scavenging table to preserve body temperature at 37°C. The right carotid artery was exposed and cannulated using PE50 tubing to monitor blood pressure via a pressure transducer (P23ID, Gould Statham, OH) connected to an IBM PC data acquisition system. The jugular vein was cannulated (PE 50 tubing) to allow for intravenous (i.v.) injections. However, due to the short half-life of various drugs, and to evade hepatic first-pass metabolism, an intra-arterial route of administration was often necessary. In animals where drugs were to be introduced by close intra-arterial (i.a.) injections, a cannula (PE 10 tubing) was inserted from the femoral artery and fed in a retrograde direction to position the tip at the level of the superior mesenteric artery.

A median laparotomy exposed the gastrointestinal segments of interest. Foil strain gauges (Showa type N11, Durham Instruments, Pickering, ON) were sequentially attached using Vet Bond glue to the gastric antrum (2 cm proximal to the pyloric sphincter); to the anti-mesenteric border of the duodenum (1-2 cm distal to the gastroduodenal junction); and lateral to the anti-mesenteric border of the ileum (just proximal to the ileo-cecal junction). All foil strain gauges were oriented parallel to the longitudinal muscle layer since this affords the most sensitive setting for recording circumferential motor activity. Wire leads from the foil strain gauges were exteriorized and attached to an IBM PC data acquisition system via a 3-channel interface box. The detailed method for recording and analyzing *in vivo* motility using foil strain gauges coupled to a computer based data acquisition system, has been described by Krantis et al. (1996). A schematic diagram of this method for recording gut

motility is shown in Figure 1. After completion of the surgery, the rats were turned over to the prone position, and Halothane was maintained at 1% for the remainder of the experiment.

*Ex vivo* organ preparations were made using the same surgical procedures described above, except rats were left in the supine position to maintain the foil gauge attachment sites exposed. This allowed for local administration of drugs directly onto the serosal surface of the gut. Regular application of warmed saline kept the exposed gut segments moist.

Gut organs for use in *in vitro* gut organ baths were prepared according to McKay and Krantis (*Can. J. Physiol. Pharmacol.*, 69: 199-204 (1991)). Rats were euthanized, and 4-5 cm segments of the proximal duodenum, jejunum, and ileum were quickly removed, carefully cleared of contents, any mesenteric attachments dissected away, and then placed in an organ bath containing Krebs solution of the following composition (mM):  $\text{Na}^+$  (151.0),  $\text{K}^+$  (4.6),  $\text{Mg}^{2+}$  (0.6),  $\text{Ca}^{2+}$  (2.8),  $\text{Cl}^-$  (134.9),  $\text{HCO}_3^-$  (24.9),  $\text{H}_2\text{PO}_4^-$  (1.3),  $\text{SO}_4^{2-}$  (0.6), and glucose (7.7). This solution was maintained at 37° C and continuously gassed with 95%  $\text{O}_2$  : 5%  $\text{CO}_2$  to give a pH 7.4.

Individual gut segments were then mounted horizontally to record circular muscle activity at two attachment points on the mesenteric border 25 mm apart, each opposite a frog heart clip tethering the segment to the bottom of the organ bath, and each connected to Grass isometric force transducers by thin polyester string. Mechanical activity detected by the transducers was monitored directly by a MacLab Macintosh data acquisition system (Apple Corp., Toronto, Ontario).

Each attachment point was placed under a resting tension of 1 gram and the segment allowed to equilibrate for 60 minutes before drug treatments. Organ bath preparations were washed with renewals of the bathing Krebs solution every 15 minutes and between drug challenges. Subsequent drug challenges were tested only after an equilibration period of at least 5 minutes or until the basal tone recovered to 90% of the resting tension.

The motor activity recorded in the *in vivo* and *ex vivo* experiments was acquired, digitized, and stored by an IBM data acquisition system that calculated, in addition to other variables, the amplitude and frequency of motor responses (Krantis et al., 1996). Qualified responses were marked depending on their capacity to satisfy a set of six numerical parameters (separately for contraction and relaxation), based on user-defined threshold values that must be satisfied within limited time periods. These parameters were continuously monitored over sequential two minute periods and adjusted as deemed necessary to

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efficaciously mark motor responses with 95-100% accuracy. The data were then output and organized into tabular form for statistical analysis.

A one-way ANOVA with a Tukey multiple analysis test was used to compare mean values, using Statgraphics Plus 5.0 software (Statistical Graphics Corp.). A probability of less than 0.05 ( $p < 0.05$ ) was regarded to be significant. All values are expressed as mean  $\pm$  S.E.M. of experiments.

All the drugs utilized in the *in vivo* and *ex vivo* experiments, including DON, were dissolved in physiological saline (0.9%). The infused concentrations (delivered at a rate of 0.5 ml/min) were:  $\alpha$ , $\beta$ -methylene adenosine triphosphate ( $\alpha$ , $\beta$ -methylene ATP, 300 mg/kg), N- $\omega$ -nitro-L-arginine methyl ester (L-NAME, 10 mg/kg), BRL 43694 (granisetron, 80 mg/kg), pentolinium ( $5 \times 10^{-5}$  M) and hexamethonium (18 mg/kg, s.c.). All drugs were purchased from Sigma Chemical Company, Toronto, ON; except for  $\alpha$ , $\beta$ -methylene ATP, which was obtained from RBI, Natick, MA; and DON was provided by Dr. Dave Miller, Agriculture Canada, Ottawa, ON, where it was biosynthetically produced and purified according to the methods of Miller et al. (*Can. J. Microbiol.*, 29: 1171-1178 (1983); Miller and Blackwell, *Can. J. Bot.*, 64: 1-5 (1986); Miller and Arnison (*Can. J. Plant Path.*, 8: 147-150 (1986)). The label "mg/kg" (or  $\text{mg}\cdot\text{kg}^{-1}$ ) refers to milligrams per kilogram of body weight of the individual subject/animal.

The concentrations of drugs applied in the organ bath preparations (*in vitro*) or topically in the *ex vivo* experiments were: carbachol (0.5 mM), papaverine (10 mM), ATP (0.5 mM), DMPP (50 mM), 3-APS (0.5 mM) (Sigma Chemical Company, Toronto, ON) and DON (20 mM). Drug volumes were never more than 1% of the bath volume.

Under control conditions, the stomach and small intestine in anesthetized rats displayed typical spontaneous motor activity. In the gastric antrum, this consisted of oscillatory contractile and relaxant motor responses. In the proximal duodenum, spontaneous motor activity was patterned into periods of intense "grouped" activity (1-5 minutes duration), comprised primarily of high amplitude, high frequency relaxations and contractions. The periods of "grouped" motor activity were separated by "intergroup" activity (3-10 minutes duration), comprised primarily of low amplitude, low frequency relaxations and contractions. In contrast to the "intergroup" activity, "grouped" activity was propagatory at a rate of  $3.4 \pm 0.6 \text{ cm}\cdot\text{min}^{-1}$  in the aboral direction. Control spontaneous

motor activity of the distal ileum consisted primarily of randomly occurring contractions and/or relaxations of relatively high amplitude and low frequency.

DON, administered systemically in a 1 or 2 mg·kg<sup>-1</sup> bolus, did not affect control motor activity. However, at 10 mg·kg<sup>-1</sup>, DON disrupted spontaneous motor patterns, as described below. Treatment with 20 mg·kg<sup>-1</sup> of DON did not yield significantly greater effects.

#### Gastric Antrum

- Within 2 minutes following intravenous injection, DON (10 mg·kg<sup>-1</sup>, i.v., n=7) inhibited antral motor activity (Figure 4), attenuating ( $p < 0.05$ ) both spontaneous contractions (to  $20 \pm 6\%$  of control) and relaxations (to  $27 \pm 11\%$  of control). This effect was transient, as motor activity recovered to 90% of the control level within  $18 \pm 3$  minutes. A proximate reapplication of DON was typically without effect.

#### Proximal Duodenum

- Within 2 minutes of systemic DON (10 mg·kg<sup>-1</sup>, n = 12) injection, spontaneous duodenal motor activity transformed from the control pattern of alternating "grouped" and "intergroup" activity to a period ( $46 \pm 15$  minutes) of sustained "grouped"-like activity (Fig. 5). This hyperactivity was not significantly different in amplitude or frequency from the control "grouped" motor activity. Within 60 minutes following the administration of DON, the control pattern of alternating "grouped" and "intergroup" activity recovered. Subsequent injection of DON (n=6) did not significantly alter the motor pattern, indicative of the development of tachyphylaxis. This tachyphylaxis to DON was relatively short-lived, such that retesting DON (n = 5) 30 minutes later, again elicited hyperactivity ( $p < 0.05$ ); however the duration of this activity ( $24 \pm 14$  min) was considerably reduced compared to the duration of the initial DON induced hyperactivity.

#### Distal Ileum

- Systemic injection of DON (10 mg·kg<sup>-1</sup>, n = 9), within 2 minutes, evoked hyperactivity of ileum. The frequency and amplitude of contractile and relaxant motor responses were significantly ( $p < 0.05$ ) increased. Thus, the pattern of gut motility in the ileum in the presence of DON resembled the characteristic fed pattern of gut motility that occurs when food has been ingested and needs to be propelled through the gut. This effect

lasted  $63 \pm 22$  minutes. Thereafter, motor activity gradually recovered to control levels. In parallel to the duodenum, tachyphylaxis to readministration of DON ( $n = 6$ ) also developed in the ileum. This tachyphylaxis persisted for up to 90 minutes, after which time, hyperactivity could again be induced by readministration of DON ( $n = 6$ ).

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#### Effects of Locally Administered DON

*Ex vivo* preparations exhibited patterned motor activity similar to that of the *in vivo* preparations. Direct (topical) application of 20 mM DON (a concentration considerably greater than the *in vivo* dose) to the serosa of the gastric antrum ( $n = 3$ ), proximal duodenum ( $n = 3$ ), or distal ileum ( $n = 3$ ) did not evoke any comparable motor responses to those seen *in vivo*. The vitality of the gut regions examined was verified by observing predictable responses to pharmacological stimuli that are known to act directly on smooth muscle, such as, papaverine (10 mM), which relaxes smooth muscle, and carbachol (0.5 mM), which induces cholinergic muscarinic receptor mediated contractions. Topically applied DON did not interfere with the action of these drugs.

Isolated gut bath preparations ( $n = 5$ ) of duodenum, jejunum, and ileum reacted with either a contraction or a relaxation to an application of carbachol (0.5 mM) or papaverine (10 mM), respectively. In addition, the gut segments exhibited relaxant responses to the putative non-adrenergic, non-cholinergic (NANC) inhibitory transmitter ATP (0.5 mM) and to neural stimulation, using the GABA<sub>A</sub> receptor agonist 3-APS (0.5 mM) or the nicotinic receptor agonist DMPP (50 mM). However, in these same preparations, DON (20 mM) was ineffective. Furthermore, DON did not interfere with the responsiveness of these gut segments to the pharmacologic agents tested.

#### Pharmacology of DON Induced Hyperactivity

L-NAME: In anesthetized rats exhibiting spontaneous motor activity, the nitric oxide (NO) synthase inhibitor, L-NAME, attenuates NO-mediated "intergroup" relaxations and enhances "grouped" activity of the duodenum (unpublished observations). Therefore, the effect of L-NAME on the action of DON was examined. Systemically administered L-NAME (10 mg·kg<sup>-1</sup>,  $n = 5$ ), did not alleviate ( $p > 0.05$ ) DON induced hyperactivity of frequency and amplitude of relaxations in the duodenum (see, Figure 6A, frequency, and Figure 6B, amplitude). In the ileum, L-NAME always enhanced both the frequency and

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amplitude of relaxations of spontaneous motor activity to the same level as with the presence of DON alone (see, Figure 6C, frequency, and Figure 6D, amplitude).

Purinoreceptor tachyphylaxis: Spontaneous duodenal "grouped" relaxations are specifically mediated through  $P_{2X}$ -receptor related purinergic transmission (unpublished observations). This was confirmed here following desensitization of the  $P_{2X}$ -purinoreceptors with prolonged exposure to the specific agonist,  $\alpha, \beta$ -methylene ATP. Initial injection of  $\alpha, \beta$ -methylene ATP ( $300 \text{ mg} \cdot \text{kg}^{-1}$ , i.a.,  $n = 3$ ) induced a prominent relaxation. Following recovery to baseline tone, re-challenge with  $\alpha, \beta$ -methylene ATP was without effect, indicative of the development of tachyphylaxis. Under these conditions, spontaneous duodenal "grouped" relaxations and ileal relaxations were specifically blocked. In addition, DON induced hyperactivity was also abolished during  $\alpha, \beta$ -methylene ATP induced tachyphylaxis in both the duodenum ( $n = 8$ ) and ileum ( $n = 4$ ).

Nicotinic receptors: Cholinergic nicotinic mechanisms are fundamentally involved in the control of intestinal motility (see, Furness and Costa, *Neurosci.*, 5: 1-20 (1980); Gershon, *Ann. Rev. Neurosci.*, 4: 227-272 (1981)). *In vivo*, treatment with the ganglionic nicotinic antagonists, pentolinium (50 mM topical application,  $n = 2$ ) or hexamethonium (18 mg/kg, s.c.,  $n = 2$ , not shown), significantly reduced the frequency and amplitude of the DON induced hyperactivity in the duodenum and ileum.

Granisetron: In the duodenum, systemically administered 5-HT<sub>3</sub> receptor antagonist granisetron ( $80 \text{ mg} \cdot \text{kg}^{-1}$ ,  $n = 8$ ) attenuated ( $p < 0.05$ ) the frequency and amplitude of spontaneous contractions and relaxations of the "grouped" activity (compare broadly spaced diagonal bars (granisetron alone) with open bars of control grouped activity in Figures 7A - 7D). This effect of granisetron persisted for up to 30 minutes. However, since the duodenum did not exhibit desensitization to the actions of this drug, granisetron was repeatedly readministered to maintain blockade of the "grouped" activity. Under these conditions, DON ( $10 \text{ mg} \cdot \text{kg}^{-1}$ ,  $n = 5$ ) consistently induced ( $p < 0.05$ ) hyperactivity (compare narrowly spaced diagonal bars (DON alone) with filled bars (granisetron + DON) in Figures 7A-7D).

Similarly in the ileum, granisetron ( $80 \text{ mg} \cdot \text{kg}^{-1}$ , i.a.) attenuated ( $p < 0.05$ ) gut motility, both spontaneous contractile and relaxant motor responses to  $40 \pm 18\%$  ( $n = 4$ ) and  $27 \pm 10\%$  ( $n = 3$ ), respectively, of control levels; however, it did not antagonize the DON induced hyperactivity (not shown).

These experiments characterized motor patterns at the level of the stomach and small intestine in anesthetized rats exposed to the mycotoxin DON. Systemic injection of DON

disrupted the gastric antrum oscillatory motor activity, replacing it with a quiescent pattern; and in the duodenum, DON induced a hyperactivity in place of the spontaneous cyclic pattern of propagatory and non-propagatory motor activity. DON also caused hyperactivity of the existing motor pattern in the ileum. The patterned activity induced by DON was reminiscent of a typical "fed pattern" motor activity. Low levels of DON were used, i.e., levels that did not induce emesis or emetic behavior. The action of DON was maximal with 10 mg/kg of DON; this dose is comparable to other studies using rodents, where up to 40 mg/kg (i.v.) of DON was used to induce alterations in feeding (Rapely et al., *Lab. Anim. Sci.*, 38: 5041 (1988)).

A progressive tolerance to DON was evident in the rats examined. DON-induced hyperactivity in the small intestine lasted up to 60 minutes, then full restoration of control motor patterns followed. Subsequently, responsiveness to routinely applied pharmacological stimuli was maintained; except for DON, which was ineffective after proximate successive applications, characteristic of the development of tachyphylaxis to DON. However, this tachyphylaxis was not sustained, possibly due to the high rate of DON detoxification in rats (Prelusky et al., *Fund. Appl. Toxicol.*, 10: 276-286 (1988)).

Many drugs, in particular emetic agents, alter intestinal motility at the level of the gut where they activate vagal afferents projecting to autonomic ganglia and/or the vomiting center of the central nervous system (CNS), which in turn reflexively stimulate the gut (Castex et al., *Brain Res.*, 688: 149-160 (1995), Cubeddu et al., *Sem. Oncol.*, 19: 2-13 (1992)). However, results from the *ex vivo* and *in vitro* experiments presented herein indicate that while the isolated gut segments were sensitive to a variety of pharmacological stimuli, directly administered DON was without effect. Thus, DON must exert its effects indirectly, from sites outside the gut. This finding aligns with certain reports in the literature of a delayed time-to-onset of DON induced effects following intragastric versus intravenous injection (30 min versus 15 min, respectively) (Coppock et al., *Am. J. Vet. Res.*, 46: 169-174 (1985), Foresyth et al., *Appl. Environ. Microbiol.*, 34: 547-552 (1977), Prelusky et al., *Natural. Toxins*, 1: 296-302 (1993)).

Under normal circumstances, feeding interrupts the fasting cyclic motor pattern at all levels of the gut, replacing it with continuous, irregular low level activity (segmentation, fed-pattern). As mentioned above, segmentation is characterized by narrow annular contractions interposed between relaxations in the small intestine, and reduced motility in the gastric antrum. The fed pattern functions to mix intestinal contents and delay antrograde propulsion



- to enhance substrate absorption (Lundgren et al., *Dig. Dis. Sci.*, 34: 264-283 (1989)). Fed pattern motility is activated by peripheral autonomic ganglia via primarily vagal inputs and is controlled, to a lesser extent, by the CNS (Chung et al., *Can. J. Physiol. Pharmacol.*, 70: 1148-1153 (1992), Tanaka et al., *J. Surg. Res.*, 53: 588-595 (1996), Yoshida et al., *J. Pharmacol. Exp. Therap.*, 256: 272-278 (1991)). Over-activation of autonomic nerves accelerates the onset and increases the duration of the fed pattern, concurrently increasing the frequency and amplitude of propagatory motor activity (Hall et al., *Am. J. Physiol.*, 250: G501-G510 (1986), Johnson et al., *Am. J. Surg.*, 167: 80-88 (1994)). This motor activity is similar to the DON induced hyperactivity in the small intestine, observed above. In addition,
- 10 DON induced inhibition of antral motor activity and delay of gastric emptying (Fioramonti et al., *J. Pharmacol. Exp. Therap.*, 266: 1255-1260 (1993)), is also characteristic of the fed pattern (Hall et al., 1986). Taken together, these results indicate that DON, from extrinsic sites, stimulates pathways mediating the fed pattern, either via peripheral autonomic ganglia or vagal efferents.
- 15 Fed pattern motility can be partially activated by the suppression of inhibitory nervous influences (Lundgren et al., 1989). Hence, DON, acting outside the gut, could stimulate hyperactivity by eliminating the tonic suppression of the enteric neural circuits controlling gut motility. NO (nitric oxide) is proposed to be a tonically released inhibitory mediator that modulates gastrointestinal motility (Daniel et al., *Am. J. Physiol.*, 266: G31-G39 (1994),
- 20 Gustafsson et al., *J. Aut. Nerv. Sys.*, 44: 179-187 (1993), Hryhorenko et al., *J. Pharmacol. Exp. Therap.*, 271: 918-926 (1994)). In the *in vivo* experiments, treatment with the NO synthesis inhibitor L-NAME (10 mg/kg, i.v.) mimicked to some degree the effects of DON, by potentiating specific motor activity of the duodenum and ileum. However, L-NAME treatment did not affect DON actions in the gut.
- 25 These experiments provide valuable insights into the pathways mediating DON effects in the gut. While the NO synthase inhibitor, L-NAME, selectively blocked spontaneous "intergroup" duodenal relaxations, and potentiated grouped motor activity, it did not affect DON induced hyperactivity. By contrast,  $\alpha, \beta$ -methylene ATP induced tachyphylaxis, which selectively attenuated "grouped" relaxations, also prevented DON
- 30 induced hyperactivity in the duodenum. There is strong evidence suggesting that ATP and NO are NANC inhibitory neurotransmitters in the rat duodenum (Katsuragi et al., *J. Pharmacol. Exp. Therap.*, 259: 513-518 (1991), Manzini et al., *Eur. J. Pharmacol.*, 123: 229-236 (1986), Postorino et al., *J. Auton. Pharmacol.*, 15: 65-71 (1995), Windschief et al., *Br.*

*J. Pharmacol.*, 115: 1509-1517 (1995)). This targeting of purinergic relaxations by DON was also apparent in the ileum, where DON induced hyperactivity was blocked by  $\alpha$ ,  $\beta$ -methylene ATP tachyphylaxis. Until now, the identity of the transmitter mediating spontaneous ileal relaxations *in vivo* has not been examined in the rat. However, there is a large body of *in vitro* functional evidence for both ATP and NO to mediate NANC relaxations in the rat ileum (Belai et al., *Cell. Tiss. Res.*, 278: 197-200 (1994), Fargeas et al., *Gastroenterol.*, 102: 157-162 (1992), Mahmood and Huddart, *Comp. Biochem. Physiol.*, 106C: 79-85 (1993), Smits et al., *Br. J. Pharmacol.*, 118: 695-703 (1996)).

- Nicotinic receptor blockade abolished both spontaneous and DON induced motor activity in the small intestine, which explains results from a several previous studies. Nicotinic ganglionic transmissions are known to mediate cholinergic stimulation of both excitatory and inhibitory intramural neurons that modulate and process enteric neural signals (Bornstein et al., *Clin. Exp. Pharmacol. Physiol.*, 21: 441-452 (1994), Gershon, *Ann. Rev. Neurosci.*, 4: 227-272 (1981)). In fact, cholinergic neurons mediate all motility patterns of the gastrointestinal tract, including the peristaltic reflex and MMCs. Therefore, treatment with nicotinic antagonists would effectively block most if not all enteric neural circuits.

- 5-HT<sub>3</sub> sites on vagal afferents are not likely to be involved in DON actions related to gut motor activity, since DON was ineffective when applied directly onto the exposed gut of a whole animal. 5-HT<sub>3</sub> receptors are also localized to myenteric neurons that are involved in the enteric circuits regulating the interdigestive motor pattern (Hoyer, *Neuropsychopharmacol.*, 3: 371-383 (1990), Yoshida et al., 1991), and these neurons may occur within the enteric pathway(s) targeted by DON. Treatment with granisetron, a potent and specific 5-HT<sub>3</sub> receptor antagonist, at a sufficiently high dose that abolished spontaneous "grouped" activity in the duodenum and motor activity of the ileum, did not affect DON-induced hyperactivity. Therefore, DON and 5-HT operate through different pathways that target common enteric elements in the small intestine. However, both the DON activated pathway and the 5-HT<sub>3</sub> receptor-dependent pathway converge on the same population of inhibitory purinergic motor neurons.

- These findings provide an explanation for DON-induced feed refusal. The effects of "low" threshold levels of DON are manifest in the disruption of gut motility; antral motor activity is diminished, and motor activity of the small intestine is intensified. Together, these motor patterns exemplify the "fed state", a state normally associated with satiation. Under these circumstances, a human or other animal ceases to eat. This DON-induced fed pattern is

transient, and the interdigestive cyclic pattern soon recovers, presumably because DON plasma levels fall below threshold.

- A consistent feature of this study, was the targeting by DON of enteric  $P_{2X}$ -purinoceptor mediated inhibitory enteric motor innervation. This innervation also involves
- 5 nicotinic receptors, but, as described above, the ubiquitous distribution and involvement of nicotinic sites within enteric neural circuits precludes the use of nicotinic antagonists to counteract the actions of DON. More promising however, is the specificity of DON in activating  $P_{2X}$ -purinoceptor related activity.  $P_{2X}$ -purinergic sites represent a highly restricted component of enteric pathways, and hence targeting these sites may represent a simple
- 10 approach for counteracting DON effects in the gut.

#### Example 2

The effects of DON on spontaneous motor activity of the gastrointestinal tract in swine *in vivo*: involvement of enteric  $P_{2X}$ -purinoceptors.

- 15 This example demonstrates that the trichothecene DON affects gut motility by acting at a site in the peripheral nervous system and that the affect of DON may be counteracted by the  $P_{2X1}$  purinoceptor desensitizing agonist,  $\alpha, \beta$ -methylene ATP, which binds with high affinity to the  $P_{2X1}$  purinoceptor on gut tissue. The intense binding by this purinergic ATP analog not only desensitizes the  $P_{2X1}$  purinoceptor regulation of gut motility from DON, but
- 20 also can shut down the pathway of regulation of gut motility effectively as an  $P_{2X1}$  purinoceptor antagonist.

- Male Yorkshire pigs (10-15 kg live weight) weaned for one week were fasted for 12 hours overnight, with free access to water. On the morning of the surgery, animals were sedated using Ketamine (8 mg/kg) via an intramuscular injection. Ketamine is a dissociative
- 25 anaesthetic, which causes an increase in blood pressure and skeletal tone, and the trachea will be stiff. A cataleptic sedation is produced with a lack of awareness of the surroundings. However, salivary secretions are increased and hence airway obstruction is a hazard; yet atropine cannot be used. Anesthesia was induced using a Halothane-oxygen mixture by application of a face mask. Topical pharyngeal anaesthesia was provided using 1-2 doses of
- 30 lidocaine aerosol (10 mg per dose, Xylocain, Sigma). Animals were then intubated, and a surgical plane of anesthesia was achieved using Halothane (3-4%) in oxygen (200 ml/min) via a closed non-rebreathing circuit. A catheter was inserted into a superficial ear vein for electrolyte replacement (0.9% saline) and intra venous drug injections. The femoral artery

was also cannulated for intra-arterial drug injections. PE 205 tubing was fed in a retrograde direction such to place the tip of the cannula at the level of the superior mesenteric artery. Blood pressure was also monitored through this arterial catheter by way of a pressure transducer (P23ID, Gould Statham, OH, USA) connected to an online IBM data acquisition system. The animals were then subjected to a laparotomy, and foil strain gauges (Showa type N11, Durham Instruments, Pickering, ON) were affixed, using Vet Bond glue as described in Example 1, onto the serosa of the gastrointestinal tract. One strain gauge was placed on the gastric antrum (5-10 cm distal to the pylorus); a second gauge was placed on the anti-mesenteric border of the proximal duodenum (2-10 cm from the pylorus), and a final gauge was attached onto the serosa of the distal ileum (2-10 cm distal to the cecum). All three foil strain gauges were oriented parallel to the axis of the longitudinal muscle. Leads from the strain gauges were exteriorized and attached to the IBM data acquisition system, via an interface box. Following completion of the surgery, the pigs were turned over to their side, and a light plane of anesthesia was maintained for the remainder of the experiment by 1-2% Halothane.

Motor activity was continuously recorded from all foil strain gauges simultaneously using data acquisition software (AD1000 analog to digital conversion card, Real Time Devices Inc., Dr. Frank Johnson, Institute of Medical Engineering, University of Ottawa) and an IBM compatible computer. Qualified motor responses were selected based on their capacity to satisfy two sets (for contractions and relaxations) of six numerical values. These values defined threshold duration and magnitude parameters that efficaciously marked motor activity based on the user's visual inspection of the recordings. The user is able to continuously monitor these parameters over sequential two minute periods and adjust the values as deemed necessary to efficaciously mark motor responses within 95-100 % accuracy. The data acquisition software output the frequency, amplitude, area, time to peak and duration of both contractile and relaxant motor responses.

A one-way ANOVA with a Tukey multiple analysis was used for comparison between mean values, using Statgraphics Plus 5.0 program. A probability of less than 0.05 ( $p < 0.05$ ) was regarded to be significant. All values are expressed as mean  $\pm$  S.E.M. of experiments.

All the drugs utilized in the *in vivo* and *ex vivo* experiments, including DON, were dissolved in physiological saline (0.9%). The infused concentrations (delivered at a rate of 0.5 ml/min) were:  $\alpha, \beta$ -methylene ATP (300  $\mu\text{g/kg}$ ), L-NAME (10 mg/kg), granisetron (80

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- $\mu\text{g/kg}$ , pentolinium ( $5 \times 10^{-5} \text{ M}$ ) and hexamethonium ( $18 \text{ mg/kg}$ , s.c.). All drugs were purchased from Sigma Chemical Company, Toronto, ON; except for  $\alpha, \beta$ -methylene ATP and methylthio ATP, which were obtained from RBI, Natick, MA; and DON was provided by Dr. Dave Miller, Agriculture Canada, Ottawa, ON, where it was biosynthetically produced and purified according to the methods of Miller and Arnison, 1986.

#### Spontaneous Motor Activity of the Gastrointestinal Tract

- Stomach:** Motor activity in the gastric antrum generally consisted of oscillatory contractions and relaxations. These were either present for the entire duration of the control recording or they occurred randomly. A motor pattern analogous to MMCs was not evident in the stomach recordings. A summary of the spontaneous motor activity is shown in Table 1.

- Duodenum:** Spontaneous motor activity consisted of an irregular pattern of contractile and/or relaxant motor activity (Table 1). Occasionally, activity reminiscent of MMCs, consisting of phase III propagatory type activity ("grouped" activity) and quiescent periods, were evident. The duration of the "grouped" activity was approximately 5 minutes, however the cycle length could not be accurately determined, since the "grouped" activity did not arise more than 2 or 3 times during the control period, which only lasted up to 2 hours in our experiments. MMCs in fasted pigs are known to have a cycle length of 70-115 minutes.
- In our pigs, the "grouped" activity consisted of relatively high amplitude, high frequency relaxations and contractions: frequency of contractions:  $11.9 \pm 0.5 \text{ events/min}$ ; amplitude of contractions:  $0.08 \pm 0.01 \text{ g}$ ; frequency of relaxations:  $12.9 \pm 0.8 \text{ events/min}$ ; amplitude of relaxations:  $0.07 \pm 0.01 \text{ g}$ .

- Ileum:** The ileum usually exhibited random contractile and/or relaxant motor activity (Table 1). MMC-like activity was rarely observed. In one third ( $n = 5$ ) of the pigs, motor activity of the ileum was in a quiescent state; however in these experiments, the ileum proved to be responsive to DON treatment.

**TABLE 1.** Characteristics of spontaneous interdigestive motor activities in anesthetized pigs

Parameters	Stomach	Duodenum	Ileum
Amp. contraction	$0.16 \pm 0.04$	$0.07 \pm 0.01$	$0.05 \pm 0.01$
Amp. relaxation	$0.19 \pm 0.03$	$0.07 \pm 0.01$	$0.05 \pm 0.01$
Freq. contraction	$4.8 \pm 0.5$	$6.7 \pm 0.6$	$3.2 \pm 0.5$
Freq. relaxation	$5.5 \pm 0.3$	$6.8 \pm 0.5$	$3.6 \pm 0.5$

Values represent the mean  $\pm$  SEM of data obtained from 12 pigs.  
Amp: Amplitude (grams tension); Freq: Frequency (events/min.); Dur:  
Duration (seconds).

- 5 DON was administered at 0.1 mg/kg (n = 3), 0.7 mg/kg (n = 2) and 1.0 mg/kg (n = 10), via either an intravenous (i.v.) or intra-arterial (i.a.) route. Within 5 minutes following injection, DON (n = 6) decreased ( $p < 0.05$ ) the frequency and amplitude of the spontaneous contractile and relaxant motor responses. The duration of this DON induced inhibition lasted from 10 minutes up to an indefinite period of time. By contrast, in three pigs, DON increased
- 10 ( $p < 0.05$ ) the frequency and amplitude of the spontaneous motor activity by  $182 \pm 40\%$  and  $206 \pm 38\%$  respectively; this effect lasted up to 30 minutes before recovering to the control pattern. This differential action of DON was not obviously correlated to either the dose injected or the route of administration. Furthermore, neither the dose injected nor the route of administration induced any changes to the mean arterial blood pressure, which continued a
- 15 steady level for the duration of the control and DON treatment periods.

- The effects of DON were more consistent in the duodenum (n = 21), where it always potentiated ( $p < 0.05$ ) the spontaneous motor activity. A systemically administered dose of DON greater than or equal to 1 mg/kg consistently induced significant potentiation; it also represents the dose which increased the frequency, as well as the amplitude, of duodenal
- 20 motor activity. The frequency of the DON induced hyperactivity typically remained elevated for the entire duration of the experiment, while the amplitude of the motor responses progressively recovered to control levels.

- Dose effects of DON on frequency and amplitude parameters of contractile and relaxant motor activity were examined for dosages of 0.1 mg/kg, n = 3; 1.0 mg/kg, n = 12; 10
- 25 mg/kg, n = 3. A clear dose-response effect was evident only for the amplitude of the motor activity. In addition, enhanced motor activity due to DON at 10 mg/kg was not significantly different from the effects of DON at 1 mg/kg.

- Systemically administered DON induced a dose-dependent increase in the ileal motor activity. DON was given at doses of 0.1 mg/kg (n = 3); 0.7 mg/kg (n = 3); 1.0 mg/kg (n =
- 30 12); and 10 mg/kg (n = 4). In the ileum, the dose-response effect was evident in both frequency and amplitude parameters of the motor activity. However, maximal effects of DON occurred at a dose equal to or greater than 1 mg/kg, where both the frequency, as well as the amplitude, of the contractile and relaxant spontaneous motor activity were significantly ( $p < 0.05$ ) increased. Thirty to sixty minutes following the DON enhanced activity, the

frequency and amplitude of the present motor activity started to decrease, however, two hours after the initial injection of DON, the motor activity was still significantly higher than control.

5 Effects of  $\alpha,\beta$ -methylene ATP against DON Induced Hyperactivity

$\alpha,\beta$ -methylene ATP was always administered during the DON induced hyperactivity. This afforded an internal control for DON action. At the dose of 300  $\mu\text{g/kg}$ ,  $\alpha,\beta$ -methylene ATP, given intra-arterially, induced only a transient (less than 1 minute) increase in the mean arterial blood pressure.

- 10 Administration of  $\alpha,\beta$ -methylene ATP (300  $\mu\text{g/kg}$ , i.a.) always induced an initial relaxant response in the stomach, however, it did not counteract the effects of DON on gastric motor activity.

- Upon injection,  $\alpha,\beta$ -methylene ATP (175  $\mu\text{g/kg}$ , i.a.) usually induced a small phasic relaxation of the duodenum. However, DON induced hyperactivity did not appear to be affected. A higher dose of  $\alpha,\beta$ -methylene ATP (300  $\mu\text{g/kg}$ , i.a.) more consistently induced an initial phasic relaxation ( $0.5 \pm 0.1$  g,  $n = 10$ ), with subsequent transient (3-10 minutes) reduction in the DON induced hyperactivity.  $\alpha,\beta$ -methylene ATP significantly decreased the amplitude, but not the frequency, of the DON induced relaxations and contractions (see, Fig. 8). When  $\alpha,\beta$ -methylene ATP (300  $\mu\text{g/kg}$ ,  $n = 3$ ) was readministered 10-20 minutes following the initial injection, the duodenum again relaxed. However, there appeared to be no further effect on the DON induced hyperactivity.

- Analogous results were observed in the ileum, where  $\alpha,\beta$ -methylene ATP (300  $\mu\text{g/kg}$ , i.a.) induced a large phasic relaxation ( $1.2 \pm 0.2$  g,  $n = 6$ ) upon injection, and reduced DON induced hyperactivity. The efficacy of  $\alpha,\beta$ -methylene ATP in reducing the amplitude, as well as the frequency, of DON induced relaxations and contractions in the ileum is presented in Figure 9. In three trials,  $\alpha,\beta$ -methylene ATP (300  $\mu\text{g/kg}$ ) was readministered within 10 minutes of the initial dose, to test for the development of tachyphylaxis. The amplitude of the relaxation to  $\alpha,\beta$ -methylene ATP was reduced by  $68 \pm 18\%$  compared to the initial administration of this agent.

## Example 3

Non-adrenergic, non-cholinergic (NANC) control of interdigestive motor activity in the rat small intestine, *in vivo*.

This example provides a study of the neural pathways controlling interdigestive motor activity in different regions of the rat small intestine *in vivo*. The data in this example, along with the data in Examples 1 and 2, above, indicate that modulation of gut motility is being controlled by neural circuitry as shown in the schematic representations of Figures 2 and 10.

The migrating motor complex (MMC) is associated with interdigestive propulsion of intestinal contents, and like peristalsis, involves sequential activation of excitatory and inhibitory pathways. The neural circuitry underlying peristalsis comprises excitatory (primarily cholinergic) and inhibitory non-adrenergic, non-cholinergic (NANC) motor neurons innervating the gastrointestinal smooth muscle, as well as excitatory and inhibitory interneurons (Costa and Brookes, *Am. Gastroenterol.*, 89: S129-S137 (1994)). However, little was previously known about the intramural neurons controlling interdigestive motility, mainly since MMCs are not easily assessed *in vitro*. Moreover, analysis of MMCs *in vivo* has for the most part focussed on the contractile activity only. *In vivo* motility studies such as those described herein revealed that propagatory intestinal motor activity, characteristic of MMCs, consist of contractions as well as relaxations.

We determined the extent of cholinergic and 5-HT involvement, as well as the role of ATP, VIP and NO, in spontaneous motor activity of the duodenum and ileum.

Male Sprague-Dawley rats (250-350 g) were fasted for 24 hrs with free access to water. For surgery, anesthesia was induced with 2% Halothane in 500 ml/min oxygen, and body temperature was maintained constant at 37°C using a thermostatically controlled heated table and a thermal blanket. The right carotid artery was exposed and cannulated to monitor blood pressure via a pressure transducer (P23ID, Gould Statham, OH). The right jugular vein was cannulated for intravenous drug injections. An intra-arterial (i.a.) route of drug administration was often favored, due to the short half-life of many drugs and to evade hepatic first-pass metabolism. For this, a cannula was inserted from the right femoral artery and fed in a retrograde direction to position the tip at the level of the superior mesenteric artery.

Animals were prepared for assessment of motility *in vivo* as described above. Foil strain gauges were sequentially attached using Vet Bond glue onto the anti-mesenteric border



of the duodenum, 1-2 cm distal to the gastroduodenal junction, and lateral to the anti-mesenteric border of the ileum, just proximal to the ileocecal junction. In 6 rats, 2 or 3 foil strain gauges were attached 2 cms apart to the proximal duodenum. From these experiments, we extrapolated the propagation velocity of the "grouped" activity. All intestinal placed foil strain gauges were oriented parallel to the longitudinal muscle layer since this affords the most sensitive setting for recording circumferential motor activity. Rats were allowed to recuperate from the surgery for one hour, then control motor activity was recorded for another hour before the administration of any drugs. All surgical and experimental protocols were carried out according to the Canadian Council on Animal Care guidelines administered by the Animal Care Committee at the University of Ottawa.

Data acquisition and statistical analysis was carried out as described above.

All the drugs were dissolved in 0.5 ml of physiological saline (0.9%). The doses (delivered within 1 minute) were:  $\alpha$ ,  $\beta$ -methylene ATP (300  $\mu\text{g/kg}$ ), methyl-S ATP (360  $\mu\text{g/kg}$ ), N- $\omega$ -nitro-L-arginine methyl ester (L-NAME, 10 mg/kg), vasoactive intestinal peptide (VIP, 4-10  $\mu\text{g/kg}$ ), BRL 43694 (granisetron, 80 mg/kg), atropine (4-6 mg/kg) and hexamethonium (18 mg/kg s.c.), all purchased from Sigma, except for  $\alpha$ ,  $\beta$ -methylene ATP and methyl-S ATP, which were obtained from RBI; granisetron which was a gift from Dr. R.K. Harding.

Region specific patterns of spontaneous motor activity were easily characterized in the rat small intestine. In the duodenum ( $n = 8$ ), this consisted of reoccurring cycles of propagating "grouped" and non-propagating "intergroup" motor activities, with a cycle length of  $5.4 \pm 0.4$  min. "Grouped" activity was typified by an intense period (approximately 2-4 min) of contractile and/or relaxant motor activity that propagated caudally at a rate of  $3.4 \pm 0.6 \text{ cm} \cdot \text{min}^{-1}$ , in a manner reminiscent of MMCs. The "intergroup" activity consisted of randomly occurring, low amplitude, low frequency relaxations and/or contractions.

Spontaneous motor activity of the ileum consisted of either relaxations (50% of all animals tested) or contractions only (30% of all animals tested); in the remaining experiments, contractile and relaxant motor activity occurred together. The predominance of one type of motor response (contraction vs. relaxation) is thought to be indicative of the intrinsic tone of the smooth muscle; where tissue with high tone shows mainly relaxant activity, whilst tissue with low tone more readily shows contractions. Generally, the spontaneous ileal relaxations and contractions occurred at a relatively low frequency, and

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were of relatively high amplitude. In 10% of the experiments, the ileum displayed periodic bursts of high frequency motor responses comparable to phase III MMC activity.

- 5 The substituted derivatives of ATP,  $\alpha$ ,  $\beta$ -methylene ATP and methyl-S ATP, have differential affinities for  $P_{2X}$ - and  $P_{2Y}$ -purinoceptors, respectively (Burnstock and Kennedy, *Gen. Pharmacol.*, 16: 433-440 (1985)). Tissues develop tachyphylaxis following prolonged exposure to these agents, and thus in this manner it was possible to discriminate between  $P_{2X}$  and  $P_{2Y}$  receptor-mediated responses. Upon injection,  $\alpha$ , $\beta$ -methylene ATP (300  $\mu$ g/kg, i.a.) evoked a phasic relaxation in the duodenum ( $1.0 \pm 0.1$  g,  $n = 5$ ); subsequently, it selectively attenuated ( $p < 0.05$ ) the frequency and amplitude of "grouped" relaxations by  $73 \pm 7\%$  and  $48 \pm 5\%$ , respectively. The effects of  $\alpha$ , $\beta$ -methylene ATP on spontaneous duodenal
- 10 contractions were variable and could not be analyzed.

- In the ileum,  $\alpha$ , $\beta$ -methylene ATP (300  $\mu$ g/kg, i.a.,  $n = 8$ ) evoked an initial phasic relaxation. Proximate rechallenge with  $\alpha$ , $\beta$ -methylene ATP did not elicit another response, indicative of the development of tachyphylaxis. During this period, spontaneous ileal
- 15 relaxations were ( $p < 0.05$ ,  $n = 8$ ) attenuated for up to 30 minutes. Spontaneous contractions were not affected by  $\alpha$ , $\beta$ -methylene ATP treatment. Methyl-S ATP (360  $\mu$ g/kg, i.a.,  $n = 4$ ) also attenuated ( $p < 0.05$ ) ileal relaxations, however, methyl-S ATP did not evoke an initial phasic relaxation.

- L-NAME (10 mg/kg, i.v.,  $n = 8$ ), selectively attenuated the frequency and amplitude
- 20 of spontaneous "intergroup" relaxations of the duodenum  $44 \pm 8\%$  and  $66 \pm 1\%$  respectively. In the ileum, L-NAME potentiated both the contractile ( $n = 6$ ) and relaxant ( $n = 8$ ) motor activity. This effect often persisted for the entire duration of the experiment. The relaxations potentiated by L-NAME were attenuated ( $p < 0.05$ ,  $n = 4-6$ ) by either  $\alpha$ , $\beta$ -methylene ATP (by  $59 \pm 12\%$ ) or methyl-S-ATP treatment (by  $70 \pm 3\%$ ).

- 25 Spontaneous contractions and relaxations of "grouped" and "intergroup" motor activity, as well as motor activity of the ileum, were all attenuated ( $p < 0.05$ ,  $n = 4$ ) for up to 20 minutes by the nicotinic receptor antagonist hexamethonium. L-NAME-enhanced activity was also attenuated ( $p < 0.05$ ,  $n = 6$ ) by hexamethonium. Atropine (4-6 mg/kg, i.a.,  $n = 4$ ), attenuated the spontaneous ileal contractions by  $87 \pm 3\%$  and  $89 \pm 7\%$  respectively.
- 30 Duodenal contractions were similarly affected.

VIP (4-10  $\mu$ g/kg, i.a.) evoked a phasic relaxation ( $n = 8$ ) in the duodenum. Subsequently, VIP transiently inhibited ( $p < 0.05$ ) duodenal "intergroup" motor activity, and

potentiated ( $p < 0.05$ ) the "grouped" activity. In the ileum, VIP consistently evoked only a slow contraction which recovered to control level within 6 minutes. Concomitant with this contraction, spontaneous ( $n = 4$ ) and L-NAME-enhanced ( $n = 6$ ) relaxations were attenuated ( $p < 0.05$ ) for up to 8 minutes. The frequency and amplitude of the spontaneous relaxations were reduced to  $33 \pm 8\%$  and  $21 \pm 5\%$  of control, respectively. The frequency and amplitude of the L-NAME induced relaxations were reduced to  $32 \pm 12\%$  and  $14 \pm 3\%$  of control, respectively.

Within 5 minutes, granisetron ( $80 \mu\text{g}\cdot\text{kg}^{-1}$ , i.v. or i.a.) attenuated ( $p < 0.05$ ) spontaneous duodenal "grouped" relaxations ( $n = 9$ ) and contractions ( $n = 4$ ), but did not affect the "intergroup" motor activity. The "grouped" motor activity was reduced for up to 40 minutes; subsequently, the control pattern of interdigestive motility progressively recovered. Granisetron treatment also attenuated ( $p < 0.05$ ,  $n = 4$ ) the spontaneous ileal contractions and relaxations. The interdigestive motor pattern of the ileum progressively recovered to control levels within approximately 60 minutes. The amplitude of the L-NAME enhanced ileal motor activity was also attenuated ( $p < 0.05$ ,  $n = 6$ ) by  $76 \pm 8\%$ , in the presence of granisetron.

Grouped relaxations were sensitive to  $\alpha, \beta$ -methylene ATP treatment, while "intergroup" relaxations were inhibited in the presence of the NO-synthase inhibitor L-NAME. By contrast, our results show that NO is not the mediator of spontaneous ileal relaxations. Others have shown that in isolated rat ileal preparations, application of ATP evokes relaxations and ATP-desensitization reduces these relaxations (Smits et al., *Br. J. Pharmacol.*, 118: 695-703 (1996)). In this study, systemic injection of the  $P_{2X}$ -purinoceptor agonist  $\alpha, \beta$ -methylene ATP, induced an initial relaxation of the ileum. A proximate re-administration of  $\alpha, \beta$ -methylene ATP did not induce a response, indicative of the development of tachyphylaxis. Concomitant with the induced tachyphylaxis, spontaneous ileal relaxations were inhibited. Hence, ATP, via  $P_{2X}$ -sites, is the transmitter mediating spontaneous NANC relaxations in the rat ileum.

ATP exhibits multiple enteric neural functions, since in addition to mediating  $P_{2X}$ -purinoceptor dependent relaxations in the duodenum and ileum, ATP via  $P_{2Y}$ -purinoceptors can stimulate NO-mediated non-propagating "intergroup" relaxations in the duodenum (Glasgow et al., *Am. J. Physiol.*, 276 (Gastrointest. Liver Physiol., 38): G889-G896 (1998)). In the present example, the  $P_{2Y}$ -purinoceptor agonist, methyl-S-ATP, inhibited spontaneous ileal relaxations. However, in contrast to  $\alpha, \beta$ -methylene ATP, methyl-S-ATP did not evoke an ileal relaxation upon injection. This indicates that  $P_{2Y}$ -purinoceptors are not present on the

smooth muscle, or else are not active within the inhibitory motor innervation(s) of the ileum. The data support the view that in the rat ileum,  $P_{2Y}$ -purinoceptors are involved in the activation of pathways mediating tonic inhibition of the purinergic NANC motor neurons targeting  $P_{2X}$  purinoceptors.  $P_{2Y}$ -purinoceptors may be present on nitrergic interneurons subserving tonic inhibition, or on other interneurons within this prejunctional input. The nitrergic and purinergic interneurons may also represent the same population, since ATP and NO synthase are co-localized in myenteric neurons in the rat ileum (Belai and Burnstock, *Cell Tiss. Res.*, 278: 197-200 (1994)).

- VIP is a NANC inhibitory transmitter in numerous gut regions (Bojo et al., *Eur. J. Pharmacol.*, 236: 443-448 (1993); Mule et al., *J. Auton. Pharmacol.*, 12: 81-88 (1992). In these experiments, VIP evoked a transient relaxation in the rat duodenum. The subsequent development of tachyphylaxis to VIP inhibited the contractile and relaxant "intergroup" activity and enhanced the "grouped" motor activity. The data indicate that the initial VIP-evoked relaxations are dependent on NO and sensitive to VIP desensitization. Thus, VIPergic interneurons must be targeting direct motor innervations (the nitrergic and cholinergic motor neurons) of the "intergroup" activity, as well as the nitrergic prejunctional modulatory inputs of the "grouped" activity.

- In these experiments, treatment with VIP inhibited the spontaneous relaxations of the ileum. Since VIP did not evoke a relaxation upon injection, it is unlikely that VIPergic neurons mediate direct inhibitory input to the ileal smooth muscle (Smits et al., *Br. J. Pharmacol.*, 118: 695-703 (1996)). *In vivo* experiments in the canine ileum, suggest that VIP plays a major role in the tonic inhibition of circular muscle motor activity via an inhibitory neural action (Fox-Threlkeld et al., *Peptides*, 12: 1039-1045 (1991)). The data support the view that VIP targets the NO-dependent prejunctional modulation of the purinergic inhibitory motor innervations in both the duodenum and ileum. These purinergic motor pathways specifically generate the propagatory motor activity of the small intestine. Furthermore, VIP specifically inhibits phase III activity of MMCs, and VIP antagonists initiate phase III activity (Hellstrom and Ljung, *Neurogastroenterol. Motil.*, 8: 299-306 (1996). The conclusion is that VIP simultaneously stimulates excitatory motor inputs and inhibitory nitrergic prejunctional inputs of the purinergic motor neurons in the rat ileum.

All components of the interdigestive motor complex are dependent upon vago-sympathetic integrity (Chung et al., *Am. J. Physiol.*, 267: G800-G809 (1994); Galligan et al., *J. Pharmacol. Exp. Therap.*, 238: 1114-1125 (1986)). The cholinergic interneurons

controlling gastrointestinal motility, act via nicotinic synapses, whereas ACh (acetyl choline) actions on smooth muscle are via muscarinic receptors. Atropine inhibited all spontaneous contractions of the small intestine. The results from this study indicate that all of the spontaneous interdigestive motor activity of the duodenum and ileum is tonically driven and is sensitive to nicotinic receptor blockade.

*In vitro* studies show neuronally derived 5-HT stimulates purinergic NANC relaxations (Briejer et al., *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 351: 126-135 (1995); Briejer et al., *J. Pharmacol. Exp. Therap.*, 274: 641-648 (1995)), and cholinergic contractions (Briejer et al., *Eur. J. Pharmacol.*, 308: 173-180 (1996)). The *in vivo* results in this study show that 5-HT<sub>3</sub> receptors are involved within the motor pathways mediating the spontaneous cholinergic contractions and purinergic relaxations in the duodenum and ileum.

An arrangement of cholinergic, nitrgergic, GABAergic, purinergic and VIPergic neural elements within the proposed tonic and modulatory pathways controlling spontaneous motor activity in the rat duodenum and ileum is presented schematically in the simplified wiring diagram of Figure 10, which also shows key locations for P<sub>2X</sub> and P<sub>2Y</sub> receptors in the pathway. Stereotypic motility patterns are elicited when driver circuits activate excitatory and inhibitory motor pathways, as determined by "enteric neural programs". However, a continuous drive from inhibitory interneurons maintains myogenic activity quiescent. This coordinated inhibition and disinhibition is mediated by the inhibitory nitrgergic inputs, and it is precisely the control of these prejunctional neuronal pathways, along with the tonically active motor pathways, which generates cyclical (interdigestive) motility patterns upon an existing baseline of motor activity. The GABAergic/nitrgergic combination pathway circuit is not present in the ileum.

#### Example 4

Effects of DON and DON-based derivatives on spontaneous gastrointestinal motor activity.

This example demonstrates the ability of DON-based derivatives to induce the fed pattern of gut motility in a manner similar to DON.

DON derivatives were selected for a comparative study with DON in rats using methods described in the previous examples for testing DON and recording its effect on gut motility. One of the representative DON derivatives was 3-acetyl DON (C<sub>17</sub>H<sub>22</sub>O<sub>7</sub>). Other new DON-based derivatives were also selected for study: isopropylidene DON (i.e., 3-

hydroxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one, having the formula  $C_{18}H_{24}O_6$ , designated EN139491); isopropylidene-3-acetyl-DON (i.e., 3-acetoxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one, having the formula  $C_{20}H_{26}O_7$ , designated EN139492); DON carbonate (i.e., 3-hydroxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate, having the formula  $C_{16}H_{18}O_7$ , designated EN139494); 3-acetyl-DON carbonate (i.e., 3-acetoxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate, having the formula  $C_{18}H_{20}O_8$ , designated EN139495); 3-acetyl-DON benzylidene acetal (i.e., 3-acetoxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one, having the formula  $C_{24}H_{26}O_8$ , designated EN139496); and DON-benzylidene acetal (i.e., 3-hydroxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one, having the formula  $C_{22}H_{24}O_7$ , designated EN139497). The chemical structures of DON and these representative DON derivatives are shown in Figures 3A and 3B.

#### Synthesis of Novel DON Derivatives

##### Isopropylidene DON (EN139491):

To a solution of 50 mg (0.168 mmol) of deoxynivalenol (DON) and 70 mg of 2,2-dimethoxypropane in 2.0 ml of anhydrous acetone at 0°C was added approximately 1 mg of p-toluenesulfonic acid. The reaction mixture was stirred and allowed to warm to room temperature. The progress of the reaction was monitored by thin layer chromatography (TLC), and the reaction was judged to be complete after 5 hours. The solvent was evaporated, and the crude product was partitioned between 2 ml of water and 5 ml of ethyl acetate. The organic layer was washed with 2 ml of saturated  $NaHCO_3$ , followed by 2 ml of saturated brine, and then dried with anhydrous magnesium sulfate. The solvent was evaporated, and the residue was chromatographed on silica gel using a 6:4 mixture of ethyl acetate-hexane mixture. This yielded 38 mg (67%) of a white solid.

$^1H$  NMR analysis ( $CDCl_3$ , 200 MHz):  $\delta$ : 6.81 (m, 1H), 4.82 (m, 1H), 4.45 (s, 1H), 4.40 (m, 1H), 3.85 (bs, 2H), 3.61 (d,  $J = 8.0$  Hz, 1H), 2.91 (d,  $J = 8.0$  Hz, 1H), 1.91-2.10 (m, 2H), 1.99 (s, 3H), 1.49 (s, 3H), 1.26 (s, 3H), 0.99 (s, 3H). The peak for the OH group could not be located in this spectrum.

Mass spectroscopy data confirmed the structure of isopropylidene DON (EN139491) as shown in Figure 3A.

## Isopropylidene-3-acetyl-DON (EN139492):

This compound was prepared in 62% yield starting with 70 mg of 3-acetyl-DON and 87 mg of 2,2-dimethoxypropane. The product was obtained as a white solid after silica gel chromatography using 1:5 ethyl acetate-hexane mixture as eluent.

- 5 <sup>1</sup>H NMR analysis (acetone-d<sub>6</sub>, 200 MHz): δ: 6.67 (d, J = 8.0 Hz, 1H), 5.09 (m, 1H), 4.81 (m, 2H), 3.85 (d, J = 8.0 Hz, 1H), 3.51 (bs, 2H), 3.12 (m, 2H), 2.61 (dd, J = 8.0 Hz, 16.0 Hz, 1H), 2.01 (s, 3H), 1.99 (dd, J = 8.0 Hz, 1H, 16.0 Hz), 1.82 (s, 3H), 1.25 (s, 6H), 1.15 (s, 3H).

- Mass spectroscopy data confirmed the structure of isopropylidene-3-acetyl-DON  
10 (EN139492) as shown in Figure 3A.

## DON-7,15-carbonate (EN139494):

- Triphosgene (5 mg, 0.016 mmol) in 1 ml of CH<sub>2</sub>Cl<sub>2</sub> was added, dropwise to a solution of 10 mg (0.033 mmol) of DON and 0.015 ml of pyridine in 1 ml of anhydrous  
15 dichloromethane at -78 °C. The reaction mixture was warmed to room temperature and stirred for another 6 hours. The solvent and remaining volatile reagents were evaporated, and residue was purified by silica gel column chromatography using ethyl acetate as eluent. The yield of DON-7,15-carbonate, a white solid, was 10 mg (99 %).

- <sup>1</sup>H NMR analysis (acetone-d<sub>6</sub>, 200 MHz): δ: 6.71 (d, J = 8.0 Hz, 1H), 5.49 (s, 1H),  
20 4.81 (d, J = 8.0 Hz, 1H), 4.51 (m, 3H), 4.31 (d, J = 16.0 Hz, 1H), 3.51 (d, J = 4.0 Hz, 1H), 3.21 (m, 2H), 1.90 2.21 (m, 2H), 1.86 (s, 3H), 1.01 (s, 3H).

Mass spectroscopy data confirmed the structure of DON-7,15-carbonate (EN139494) as shown in Figure 3B.

- 25 3-acetyl-DON-7,15-carbonate (EN-139495):

This compound was prepared in 99% yield starting with 20 mg of 3-acetyl-DON, 0.023 ml of pyridine, and 10 mg of triphosgene. The product was obtained as a white solid after silica gel chromatography using 7:3 ethyl acetate-hexane mixture as eluent.

- <sup>1</sup>H NMR analysis (CDCl<sub>3</sub>, 200 MHz): δ: 6.61 (d, J = 8.0 Hz, 1H), 5.36 (m, 1H), 5.29  
30 (s, 1H), 4.49 (d, J = 8.0 Hz, 1H), 4.41 (d, J = 16.0 Hz, 1H), 4.19 (d, J = 16.0 Hz, 1H), 3.95 (d, J = 4.0 Hz, 1H), 3.20 (m, 2H), 2.39 (s, 1H), 2.12 (s, 3H), 1.92 (m, 1H), 1.92 (s, 3H), 1.12 (s, 3H).

Mass spectroscopy data confirmed the structure of 3-acetyl-DON-7,15-carbonate (EN139495) as shown in Figure 3B.

7,15-benzylidene-3-acetyl DON acetal (EN-139496):

- 5 This compound was prepared in 95 % yield starting with 20 mg of 3-acetyl-DON and 13 mg of benzaldehyde dimethyl acetal. The product was obtained as a white solid after silica gel chromatography using 4:6 ethyl acetate-hexane mixture as eluent.

<sup>1</sup>H-NMR analysis (CDCl<sub>3</sub>, 200 MHz): δ: 7.45 (m, 5H), 6.81(d, J = 8.0 Hz, 1 H), 5.39 (s, 1 H), 5.10 (m, 1H), 4.90 (s, 1H), 4.35 (d, J = 8.0 Hz, 1H), 4.31(d, J = 16.0 Hz, 1H), 3.81 (d, J = 16.0 Hz), 3.81(d, J = 16 Hz, 1H), 3.21 (m, 2H), 2.20-2.45 (m, 2H), 2.01(s, 3H), 1.91(s, 3H), 1.31(s, 3H).

Mass spectroscopy data confirmed the structure of isopropylidene-3-acetyl-DON (EN139496) as shown in Figure 3B.

- 15 7,15-benzylidene-DON acetal (EN139497):

To a solution of 25 mg (0.084 mmol) DON and 20 mg (0.126 mmol) benzaldehyde dimethyl acetal (20 mg, 0.126 mmol) in 2 ml of anhydrous acetonitrile was added approximately 1 mg of p-toluenesulfonic acid. The reaction mixture was stirred at room temperature for 2 hours, and the solvent was evaporated. The crude residue was taken in ethyl acetate (5 ml) and was washed with saturated sodium bicarbonate solution (2 ml), followed by water (2 ml). The organic layer was separated, dried over anhydrous MgSO<sub>4</sub>, and concentrated to give a crude product, which was purified by column chromatography (7:3, ethyl acetate-hexane) to afford 27 mg, (87 %) of the title compound as a white solid.

<sup>1</sup>H NMR (Acetone-d<sub>6</sub>, 200 MHz) analysis: δ: 7.45 (m, 5H), 6.75 (d, J = 8.0 Hz, 1H), 5.35 (s, 1H), 4.95 (s, 1H), 4.51 (d, J = 8.0 Hz, 1H), 4.49 (m, 1H), 4.25 (d, J = 16.0 Hz, 1H), 3.85 (d, J = 16.0 Hz, 1H), 3.45 (d, J = 5.0 Hz, 1H), 3.11 (m, 2H), 2.10 (m, 2H), 1.85 (s, 3H), 1.25 (s, 3H). The peak for the OH group could not be located in this spectrum.

Mass spectroscopy data confirmed the structure of isopropylidene-3-acetyl-DON (EN139497) as shown in Figure 3B.

30

DON administered systemically (10 mg/kg, i.v.), induced a typical fed pattern of gut motor activity in the rat gastroduodenum (see, Figure 11). DON abruptly attenuated the gastric antral (site S1) motor activity and induced a sustained hyperactivity in the duodenum



(proximal duodenal site D1). Within 60 minutes, the control motor pattern recovered. Occasionally, tricothecene-induced hyperactivity was characterized by an initial high frequency motor activity where the amplitude although larger than "intergroup" responses was smaller than the MMC motor activity. This is shown in Figure 11, where this initial period lasted 3-10 minutes; thereafter the amplitude increased to MMC levels.

3-Acetyl-DON (10 mg/kg of body weight (bw), i.v.) induced a typical fed pattern motor activity in the rat gastroduodenum (n = 4). Figure 12 shows the time to onset of action (1 min) and duration ( $40 \pm 4$  min) of effects were similar to those for DON. Figure 13 shows the effects of intravenously administered 3-acetyl DON on spontaneous motor activity in the rat gastric antrum (S1) and proximal duodenum (D2). Within 60 minutes, the control motor pattern recovered.

Within 30 seconds of intravenous injection of EN139491 (10 mg/kg bw), there developed a long lasting ( $40 \pm 1.75$  min, n = 6) hyperactivity in the duodenum and a simultaneous and parallel attenuation of motor activity in the gastric antrum, typical of the effect seen with DON. Figure 14 shows typical *in vivo* recordings of the motor activity in the rat duodenum (D1) and gastric antrum (S1) illustrating the action of the compound EN139491 on the fasting pattern of gut motor activity. The top panel of the recording shows 20 minutes of normal fasting pattern motor activity without any drug treatment. During this period, the duodenum displayed a typical pattern of low frequency spontaneous motor activity together with propagating motor activity (MMC). The gastric antrum displayed a typically rhythmic motor activity. The second panel of the recording shows activity at the time of injection with EN139491. Within 30 seconds of injection, a long lasting (40-60 min) hyperactivity in the duodenum and a simultaneous and parallel attenuation of motor activity in the gastric antrum developed. This EN139491 induced motor activity was typical of fed pattern motor activity. Recovery of fasting pattern motor activity is shown in the bottom panel of the recording in Figure 14. Figure 15 shows the recording of the effects that EN139491 had on duodenal motor activity recorded at D2 (i.e., 1.5 cm distal to the D1 strain gauge). The recording of the induction and duration of fed pattern at D2 by EN139491 as shown in Figure 15 was similar to the results recorded at duodenal site D1 as shown in Figure 14.

A closer analysis of the features of the individual relaxation and contraction components of the fed pattern of gut motor activity induced by EN139491 was also made. In this analysis, the amplitude and the frequency of the relaxation or contraction component of

MMCs and of the fed pattern induced by EN139491 at duodenal site D1 were expressed as a percent of the respective frequency and amplitude of the relaxation or contraction component observed in the normal "intergroup" activity of the fasting pattern, which serves as an internal control for each animal. The results of the analysis indicated that both amplitude (Figure 16) and frequency (Figure 17) of the relaxation component of the fed pattern induced by EN139491 were comparable to those seen in the spontaneous "grouped" MMC activity of the gut. Likewise, both amplitude (Figure 18) and frequency (Figure 19) of the contraction component of the fed pattern induced by EN139491 were comparable to those seen in the spontaneous "grouped" MMC activity of the gut. These results indicated that the fed pattern induced by EN139491 had the same features as the fed pattern induced by DON.

As in the case EN139491, within 30 seconds of intravenous injection of the DON derivative EN139492 (10 mg/kg bw), a long lasting ( $48.5 \pm 2$  min,  $n = 6$ ) hyperactivity was induced in duodenum sites D1 and D2 and a simultaneous and parallel attenuation of motor activity in gastric antrum site S1 occurred. An example of these effects on *in vivo* gut motor activity is presented in Figure 20. Figure 20 shows a typical *in vivo* recording of the motor activity at the rat duodenum D1 and D2 sites and the gastric antrum S1 site illustrating the action of EN139492 on the fasting pattern of gut motor activity. The top panel of the recording shows more than 40 minutes of normal fasting pattern motor activity in the absence of DON or a DON derivative. During this period, the duodenum displayed a typical pattern of low frequency spontaneous motor "intergroup" activity together with propagating "grouped" motor activity (i.e., "MMC"). The gastric antrum displayed a typically rhythmic motor activity. Within 30 seconds of injection, a long lasting hyperactivity in the duodenum was initiated and a simultaneous and parallel attenuation of motor activity in the gastric antrum developed.

A closer analysis of the fed pattern of gut motor activity induced by EN139492, revealed that the frequency and amplitude of relaxation and contraction components were at least comparable to the frequency and amplitude of relaxation and contraction components of the MMC activity of the gut (data not shown). Thus, as with EN139491, the DON derivative EN139492 is able to induce a fed pattern of gut motor activity that is comparable to the fed pattern induced by DON, the structural parent of EN139491 and EN139492.

By the same methods employed to study EN139491 and EN139492 above, the DON derivative compounds referred to as DON carbonate (EN139494) and 3-acetyl DON carbonate (EN139495) (see, Figure 3B) were also tested and shown to be able to induce the

fed pattern of gut motor activity at levels that are at least comparable to those induced by the structural parent DON. Intravenous injection of the tricothecene based derivative EN139495 ( $10 \text{ mg} \cdot \text{kg}^{-1}$ ) induced a typical *in vivo* fed pattern motor activity in the gut recorded from the proximal duodenum ( $D_1$ ) and gastric antrum ( $S_1$ ) of halothane-anaesthetized male Sprague  
5 Dawley rats,  $n = 4$ . The effect of EN139495 was evident within 40 seconds of injection, and the duration of action was 40-60 minutes. At  $S_1$ , contraction amplitude and frequency were reduced to  $59 \pm 8.7\%$  and  $64.25 \pm 12.0\%$  SEM of control motor activity, respectively. The gastric antral relaxation amplitude and frequency were also reduced to  $28.4 \pm 3.4\%$  and  $48.0 \pm 10.5\%$  SEM, respectively. In the intestine ( $D_1$ ), there was a profound hyperactivity:  
10 contraction amplitude and frequency were increased to  $119.0 \pm 12.0\%$  and  $1598.8 \pm 421.9\%$  SEM of control motor activity, respectively. Relaxation amplitude and frequency were also increased to  $331.0 \pm 39.8\%$  and  $724.4 \pm 180.75\%$  SEM, respectively.

In approximately 20% of experiments, the 3-acetyl-DON and EN139491 induced hyperactivity did not show an immediate large amplitude activity. An example of this is  
15 shown in Figure 15. Although there was an initial high frequency motor activity, the large amplitude motor activity was delayed, replaced by responses larger than "intergroup" responses but smaller than the MMC motor activity. When this "delay" in amplitude occurred, it usually lasted 3-10 minutes. Thereafter, the amplitude of the induced hyperactivity increased to MMC levels as shown in Figures 16-19. The initial differential  
20 action of these DON derivative compounds did not affect the duration of the induced fed pattern of gut motor activity.

A proximate reapplication of DON or its derivatives within 90 minutes of the first injection was typically without effect. After 120 minutes, DON and its derivatives were again effective and could induce the fed pattern of gut motor activity.

The foregoing results demonstrate the effectiveness of DON and a familiar derivative of DON, 3-acetylated DON (which is reported to have the lowest toxicity of all the tricothecenes), to induce a fed pattern of gut motor activity was tested. In addition, two new DON-based derivatives (EN139491 and EN139492) were synthesized and shown to be capable of inducing a fed pattern of gut motor activity in a manner at least comparable to  
30 DON. All derivatives (tested intravenously as a single 1 ml bolus at a dose of  $10 \text{ mg} \cdot \text{kg}^{-1}$ ) displayed a similar profile of action to that of a single bolus i.v. injection of DON: within 1 minute of intravenous injection in anesthetized Sprague Dawley rats, the spontaneous fasting motor pattern of the gastroduodenum changed to a typical fed pattern motor activity. In the

gastric antrum, oscillatory motor activity was replaced with a quiescent pattern; and in the duodenum, DON induced a sustained hyperactivity in place of the cyclic "grouped" MMC pattern. This effect lasted 40-60 minutes, and the spontaneous motor activity then recovered to a fasting pattern of motor activity. Neither DON nor its derivatives caused any discernible effects on blood pressure, heart rate, or respiratory rate.

#### Example 5

Effects of the selective  $P_{2X1-2X3}$  purinoceptor antagonist 2',3'-O-(2,4,6-trinitrophenyl) adenosine triphosphate (TNP-ATP).

This example demonstrates the direct involvement of the  $P_{2X1}$  purinoceptor present on the smooth muscle of gut tissue in regulating gut motor activity and the ability of the  $P_{2X1-2X3}$  purinoceptor antagonist TNP-ATP to block the  $P_{2X1}$  purinoceptor and, thereby, to inhibit the fed pattern of gut motor activity induced by DON or DON-based derivatives.

In previous examples, the pharmacology of the intrinsic motor inhibitory innervation of the rat and porcine gastroduodenum was characterized using specific nitric oxide (NO) synthesis inhibitors and inhibitors of purinoceptor-mediated responses, such as the general  $P_2$  receptor antagonist, suramin, and the general  $P_{2X}$  agonist,  $\alpha,\beta$ -methylene ATP, and the  $P_{2Y}$  agonist, methyl-thiol-ATP. Proximate re-challenge for each of these purinoceptor agonists was marked by a profound tissue tachyphylaxis, which was exploited to block the respective purinoceptor. The results showed relaxations within the patterned spontaneous motor activity of the rat proximal duodenum were differentially dependent upon either NO or ATP: duodenal "grouped" MMC relaxations were sensitive to  $\alpha,\beta$ -methylene ATP treatment, whilst "intergroup" relaxations were inhibited by treatment with the NO synthesis inhibitor L-NAME. In addition, the data showed that NO was not the mediator of spontaneous ileal relaxations. These ileal relaxations were dependent upon ATP acting via  $P_{2X}$ -purinoceptors similar to the MMC related relaxations.

The previous examples also showed that interference of either motor component effectively prevented DON-induced fed pattern of motor activity. In this example, we further investigate the hypothesis that the relaxations occurring within the DON-induced fed pattern motor activity in the intestine are mediated by the  $P_{2X1}$  purinoceptor subtype. This study employed a new purinoceptor antagonist, TNP-ATP (Lewis et al., *Br. J. Pharmacol.*, 124: 1463-1466 (1998)) which has been used *in vitro* as a subtype selective antagonist (whole tissue  $IC_{50}$  in  $\mu M$  range) to determine the role of  $P_{2X1}$  and  $P_{2X3}$  homomeric and  $P_{2X2/3}$

heteromeric purinoceptors (Virginio et al., *Mol. Pharmacol.*, 53: 969-973 (1998)).  $P_{2X3}$  receptors are reported to be expressed only on sensory neurones (Evans and Suprenant, *Semin. Neurosci.*, 8: 217-223 (1996)). This study represents the first use of the  $P_{2X1}$ -selective antagonist TNP-ATP *in vivo*. The aim was to determine the role of  $P_{2X1}$  receptors in control of patterned gastrointestinal motor behaviour and test directly the hypothesis that the  $P_{2X1}$  purinoceptor subtype mediates DON-induced hyperactivity in the gut.

Four doses of TNP-ATP were tested in the *in vivo* rat model as described above. Sprague Dawley rats were continuously monitored for blood pressure, respiratory rate, pallor, and general well being. TNP-ATP had no discernible effects on these parameters throughout an experiment, which often lasted up to 6hrs.

TNP-ATP had no effect on spontaneous gastric motor activity (data not shown). By contrast, TNP-ATP injected intravenously as a single bolus significantly and specifically affected spontaneous duodenal relaxations. At 2.5 mg/kg, the actions of TNP-ATP were barely observable. By contrast, 4.5 and 5 mg/kg appeared to be supramaximal doses and the effectiveness inconsistent. Occasionally, there were also some non-specific actions on gut motor activity at these higher doses.

TNP-ATP administered at 3.5 mg/kg was found to be reproducibly effective and specific in its actions. This was chosen for subsequent evaluation in the model. Typical *in vivo* recordings showing the effects of intravenously injected TNP-ATP ( $3.5 \text{ mg}\cdot\text{kg}^{-1}$ ) on spontaneous motor activity in the rat duodenum (at duodenal site D1) is shown in Figure 21. TNP-ATP did not evoke any response upon injection. However, within 1 minute of injection, MMC related relaxations were reduced. "Intergroup" motor activity was not significantly affected. There was recovery of MMC related relaxant motor activity to within 90% of control level within 30 minutes of TNP-ATP injection.

*In vivo* recordings showing the effects of intravenously injected TNP-ATP ( $3.5 \text{ mg}\cdot\text{kg}^{-1}$ ) on DON-induced fed pattern motor activity in the rat stomach and duodenum are presented in Figures 22 (recording duodenal site D1) and 23 (recording duodenal site D2 and gastric antral site S1). Consistent with all other experiments, TNP-ATP did not evoke any response upon injection. However, within 1 minute of injection, the effects of DON ( $10 \text{ mg}\cdot\text{kg}^{-1}$ , i.v.) were significantly reduced. This inhibitory action of TNP-ATP consisted of an initial profound effect (up to 80% inhibition) lasting approximately 5 minutes, followed by a longer period of less profound (up to 40% inhibition), but significant antagonism of DON actions.

The ability of TNP-ATP to counter DON actions is shown graphically in Figures 24-27. The bar graphs of Figures 24-27 show the effects of intravenous treatment with TNP-ATP on DON-induced relaxations and contractions recorded from the proximal duodenum (D1). The effects of TNP-ATP on the amplitude (Figure 24) and frequency (Figure 25) of DON-induced relaxations were compared to the amplitude and frequency of the relaxation component of "grouped" MMC and the control "intergroup" motor activity, which was set at 100%. Likewise, the effects of TNP-ATP on the amplitude (Figure 26) and frequency (Figure 27) of DON-induced contractions were compared to the amplitude and frequency of the contraction component of "grouped" MMC and the control "intergroup" motor activity.

A consistent feature of the TNP-ATP effect was the specific attenuation of the amplitude, but not the frequency, of DON-induced duodenal hyperactivity (compare, open bars with checkered bars in Figures 24-27). The effect of TNP-ATP on the frequency of the relaxation component of DON-induced hyperactivity in the duodenum was evident within 20 seconds of administration of TNP-ATP, and maximal attenuation of the frequency of the relaxation component was attained within 2 minutes. The DON-evoked hyperactivity resumed within 35 minutes of TNP-ATP administration and recovered to 90% of the pre-TNP-ATP administration level.

The effectiveness of the selective  $P_{2X1}$  purinoceptor antagonist TNP-ATP was evaluated. An intravenous injection of a single bolus of this antagonist rapidly and specifically attenuated MMC related relaxations; indicative of the involvement of  $P_{2X1}$  purinoceptors in gut motor activity. Intravenous injection with a single bolus injection of TNP-ATP reduced (transiently) the DON-induced fed pattern in a dose-dependent manner. These results confirm the trend of the data employing a general  $P_{2X}$  receptor antagonist.  $P_{2X}$ -purinergic sites represent a highly restricted component of enteric pathways. The results clearly show that the  $P_{2X1}$  receptor subtype mediates intrinsic purinergic inhibitory innervation of the duodenum and that blocking these receptors may represent a simple approach for counteracting DON effects in the gut. Furthermore, the data support the notion that these receptor sites are potential targets for developing agents to modify feeding behavior.

All publications cited in the text are incorporated herein by reference.

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CLAIMS

1. A method of treating obesity in a vertebrate animal comprising administering to said animal a non-toxic, gut motility-regulating amount of a trichothecene or derivative thereof.
2. The method of treating obesity according to claim 1, wherein the trichothecene or derivative thereof is selected from the group consisting of DON; nivalenol; trichothecol; trichothecin; 3-acetyl DON; 7-acetyldeoxynivalenol; 3,15-diacetyldeoxynivalenol; 4-acetyl nivalenol (fusarenol-X); 4,15-diacetyl nivalenol; isopropylidene DON; isopropylidene-3-acetyl-DON; DON carbonate; 3-acetyl-DON carbonate; 3-acetyl-DON benzylidene acetal; and DON benzylidene acetal.
3. The method of treating obesity according to claim 2, wherein the trichothecene is DON.
4. The method of treating obesity according to claim 1, wherein the trichothecene is administered orally, parenterally, intravenously, intramuscularly, or intra-arterially.
5. The method of treating obesity according to claim 4, wherein the trichothecene is administered orally.
6. The method of treating obesity according to claim 1, wherein the vertebrate animal is selected from the group consisting of primates, swine, cattle, sheep, birds, horses, cats, dogs, and rodents.
7. The method of treating obesity according to claim 1, wherein the vertebrate animal is a human.
8. A method of stimulating fed pattern of gut motility in a vertebrate animal comprising administering to said animal a non-toxic, gut motility-regulating amount of a trichothecene or derivative thereof, a trichothecene analog, or a non-desensitizing agonist of the  $P_{2X1}$  receptor.

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9. The method of claim 8, wherein the trichothecene or derivative thereof is selected from the group consisting of DON; nivalenol; trichothecolone; trichothecin; 3-acetyl DON; 7-acetyldeoxynivalenol; 3,15-diacetyldeoxynivalenol; 4-acetylnivalenol (fusarenon-X); 4,15-diacetylnivalenol; isopropylidene DON; isopropylidene-3-acetyl DON; DON carbonate; 3-acetyl-DON carbonate; 3-acetyl-DON benzylidene acetal; and DON benzylidene acetal.

10. The method of claim 8, wherein the trichothecene is DON.

11. The method of claim 8, wherein the trichothecene is administered orally, parenterally, intravenously, intramuscularly, or intra-arterially.

12. The method of claim 8, wherein the trichothecene is administered orally.

13. The method of claim 8, wherein the animal is selected from the group consisting of primates, swine, cattle, sheep, birds, horses, cats, dogs, and rodents.

14. The method of claim 8, wherein the vertebrate animal is a human.

15. The method of claim 8, wherein the non-desensitizing agonist of the  $P_{2X1}$  receptor is an analog of ATP.

16. A method of increasing weight in a vertebrate animal comprising administering to said animal an analog of ATP in an amount sufficient to inhibit fed pattern gut motor activity.

17. The method of claim 16, wherein the analog of ATP is a desensitizing agonist or an antagonist of the  $P_{2X1}$  purinoceptor.

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18. The method of claim 17, wherein the analog of ATP is selected from the group consisting of  $\alpha,\beta$ -methylene ATP and 2',3'-O-(2,4,6-trinitrophenyl)-ATP.
19. A method of preventing fed pattern of gut motility in a vertebrate animal comprising administering an analog of ATP.
20. The method of claim 19, wherein the analog of ATP is a desensitizing agonist or an antagonist of the  $P_{2X1}$  receptor.
21. The method of claim 20, wherein the analog of ATP is selected from the group consisting of  $\alpha,\beta$ -methylene ATP and TNP-ATP.
22. A method of identifying a compound for treating obesity comprising determining whether the compound is capable of inducing fed pattern gut motor activity.
23. The method of identifying a compound for treating obesity according to claim 22, wherein the compound is tested for the ability to induce fed pattern gut motor activity using an *in vitro* gut organ bath assay, an *ex vivo* gut organ assay, or an *in vivo* assay for gut organ motor activity.
24. The method of claim 22, wherein the fed pattern gut motor activity induced by the compound is compared to the fed pattern gut motor activity induced by DON.
25. A pharmaceutical composition for inducing fed pattern gut motor activity comprising:
- (a) a compound selected from the group consisting of nivalenol; 4-deoxynivalenol; trichothecolone; trichothecin; 3-acetyldeoxynivalenol; 7-acetyldeoxynivalenol; 3,15-diacetyldeoxynivalenol; 4-acetyl nivalenol (fusarenon-X); 4,15-diacetyl nivalenol; 3-hydroxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate; 3-acetoxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate; 3-acetoxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one; 3-hydroxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one; 3-hydroxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one; 3-acetoxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one; and combinations thereof, and
  - (b) a pharmaceutically acceptable carrier.

26. The compound 3-hydroxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one.
27. The compound 3-acetoxy-7,15-isopropylidene-12,13-epoxy-9-tricothecin-8-one.
28. The compound 3-hydroxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate.
29. The compound 3-acetoxy-12,13-epoxy-9-tricothecin-8-one-7,15 carbonate.
30. The compound 3-acetoxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one.
31. The compound 3-hydroxy-7,15-benzylidene-12,13-epoxy-9-tricothecin-8-one.

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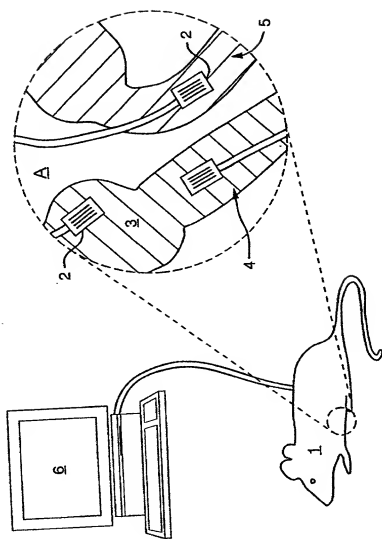


FIG. 1

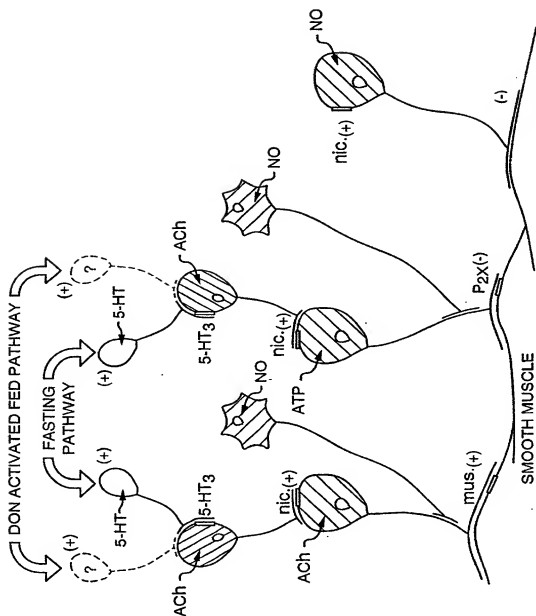
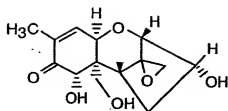


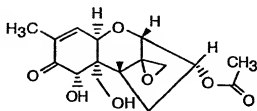
FIG. 2

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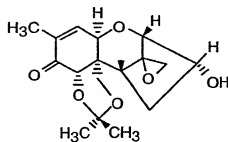
FIG. 3A



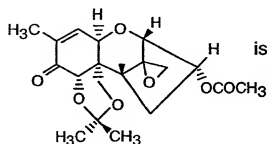
DON  
(C<sub>15</sub>H<sub>20</sub>O<sub>6</sub>)



3-acetyl DON  
(C<sub>17</sub>H<sub>22</sub>O<sub>7</sub>)



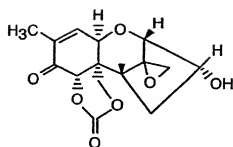
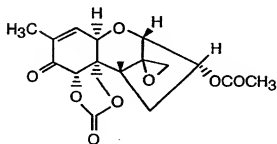
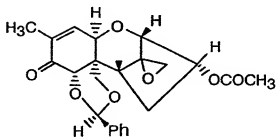
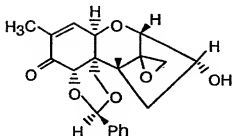
isopropylidene  
DON  
(C<sub>18</sub>H<sub>24</sub>O<sub>6</sub>)



isopropylidene-3-acetyl DON  
(C<sub>20</sub>H<sub>26</sub>O<sub>7</sub>)

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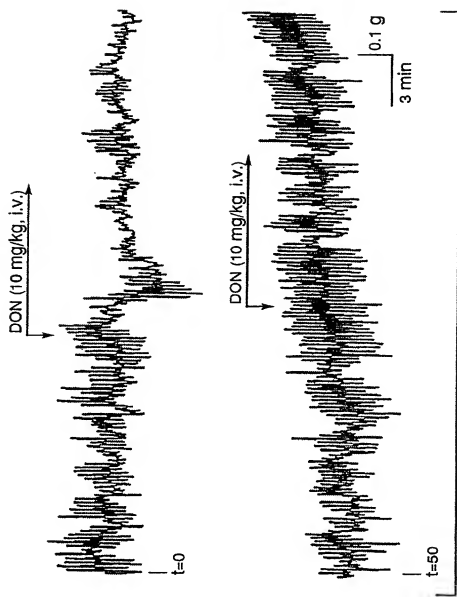
FIG. 3B

DON carbonate  
( $C_{16}H_{18}O_7$ )3-acetyl-DON  
carbonate  
( $C_{18}H_{20}O_8$ )3-acetyl-DON  
benzylidene acetal  
( $C_{24}H_{26}O_8$ )DON  
benzylidene acetal  
( $C_{22}H_{24}O_7$ )

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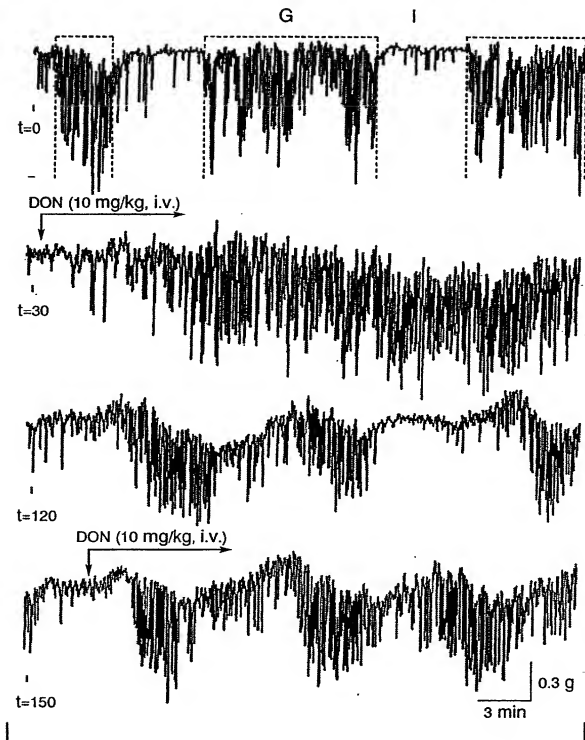


FIG. 5

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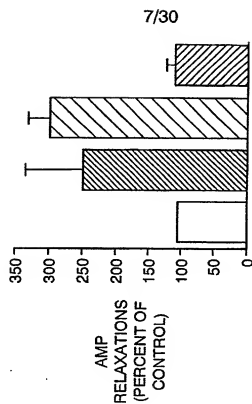


FIG. 6B

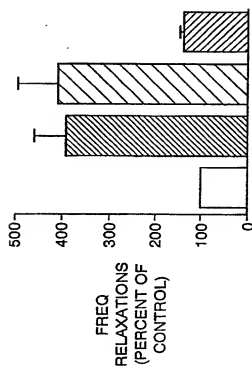


FIG. 6A

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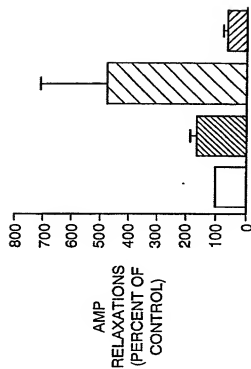


FIG. 6D

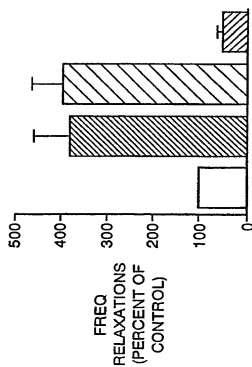


FIG. 6C

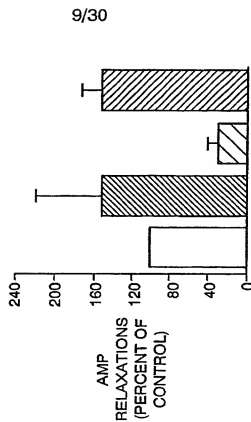


FIG. 7B

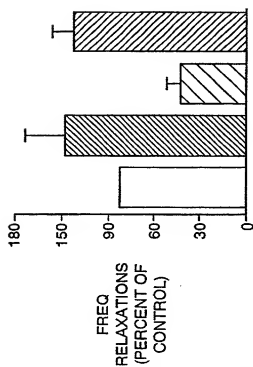


FIG. 7A

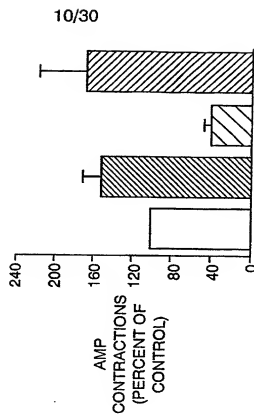


FIG. 7D

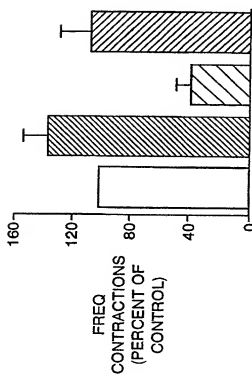


FIG. 7C

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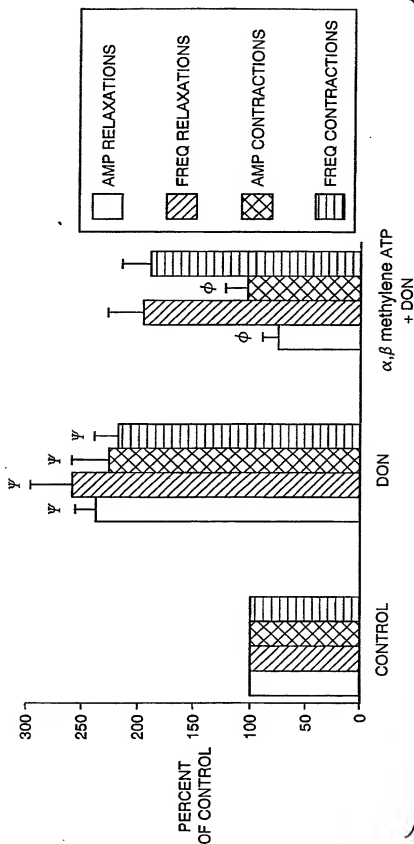
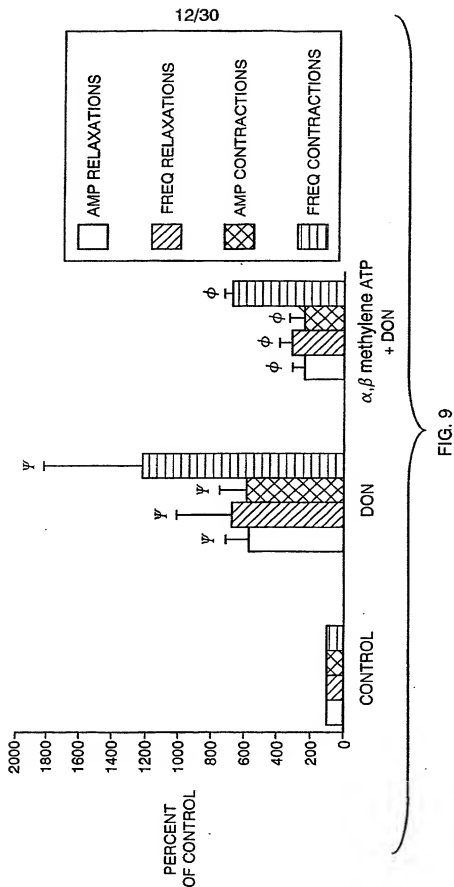


FIG. 8



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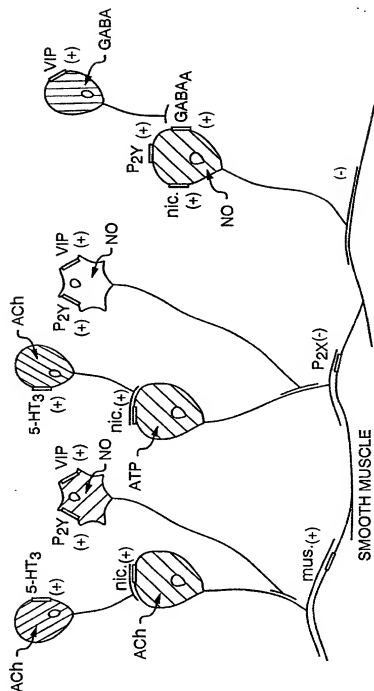


FIG. 10

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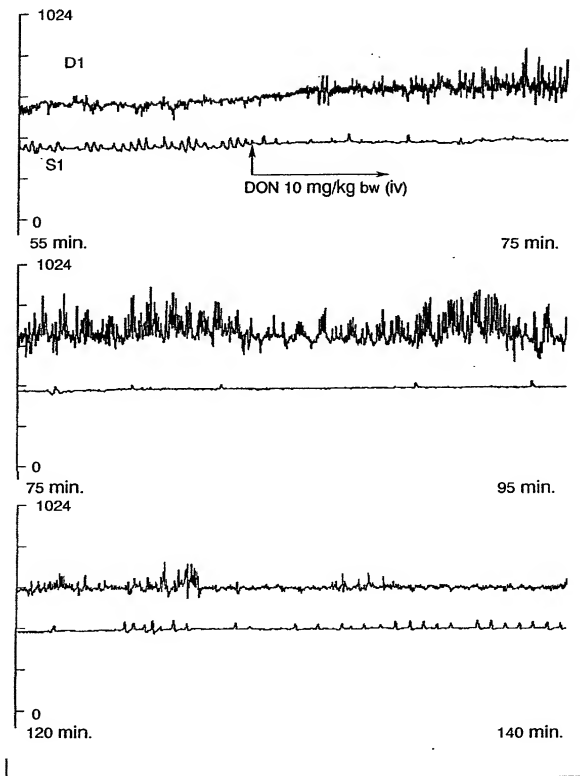


FIG. 11  
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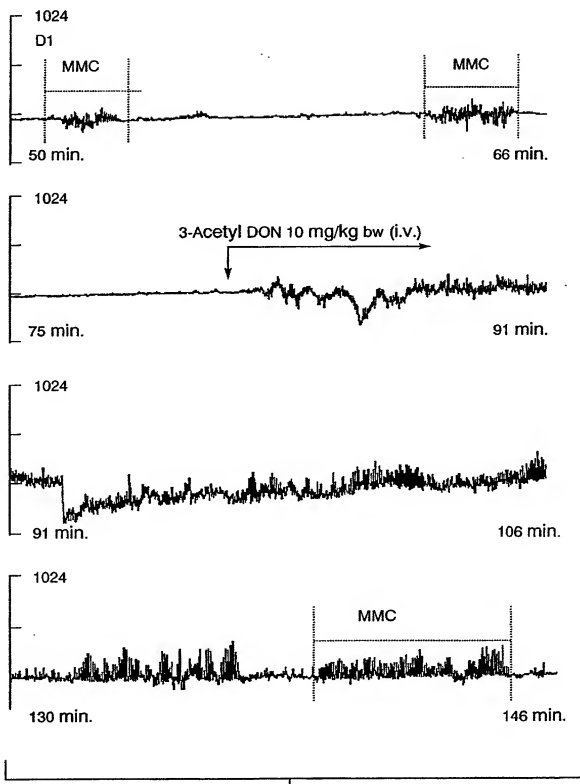


FIG. 12  
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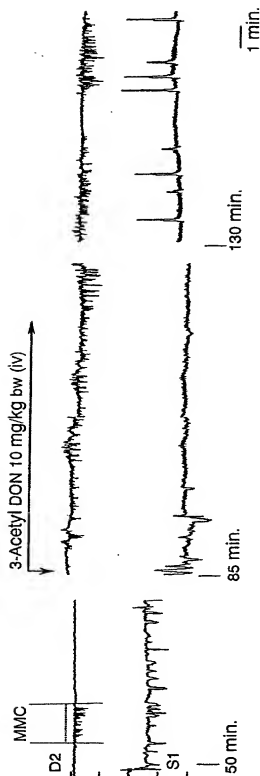


FIG. 13

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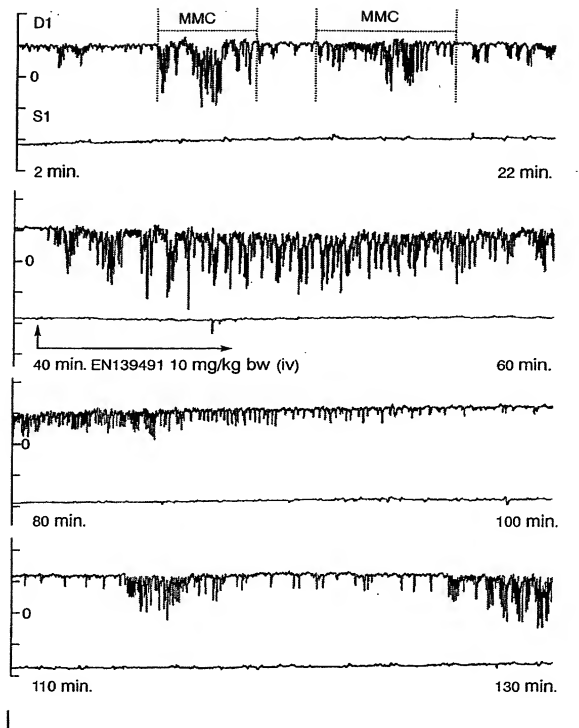


FIG. 14  
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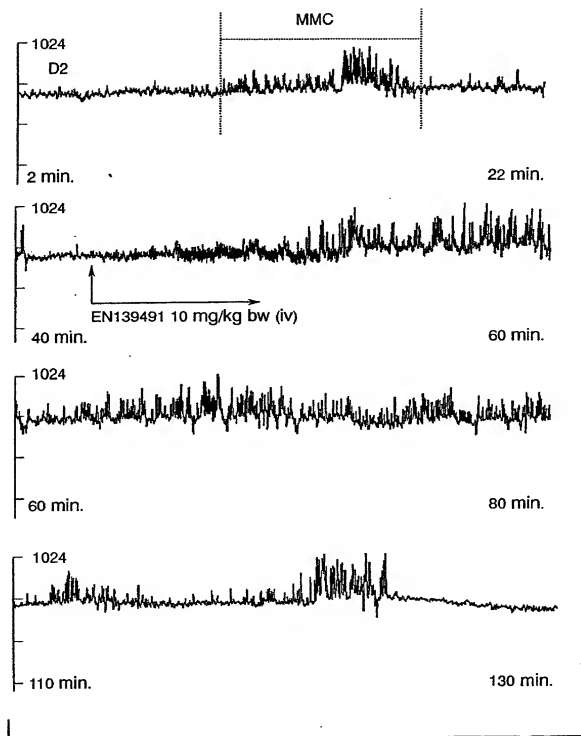


FIG. 15  
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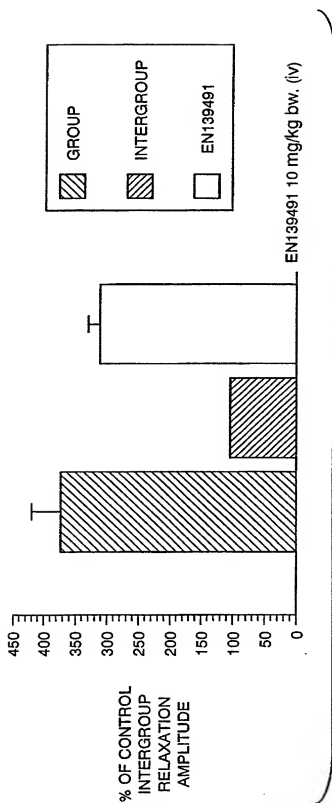


FIG. 16

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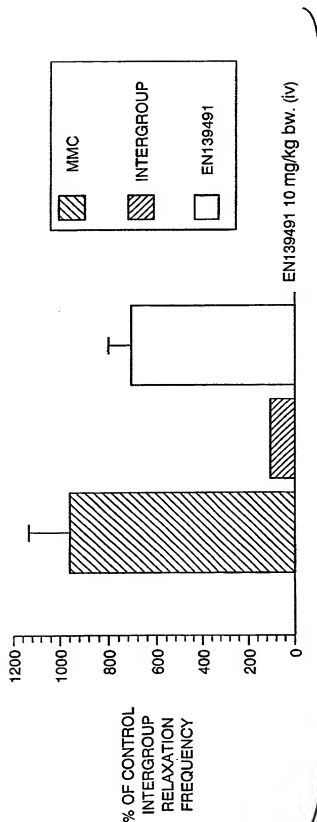


FIG. 17

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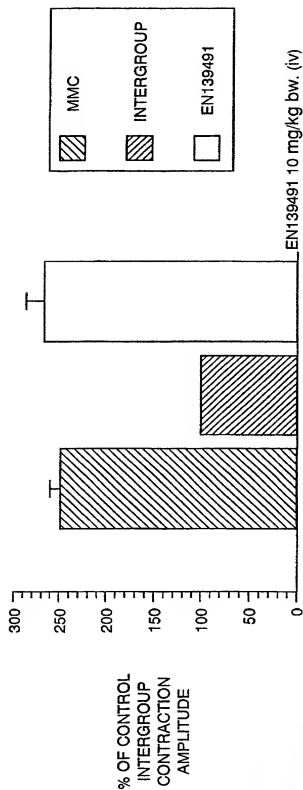


FIG. 18

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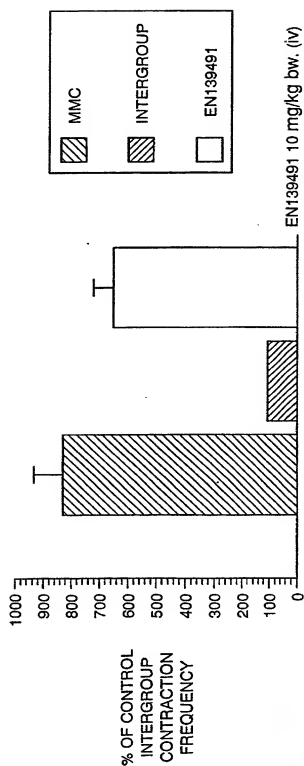


FIG. 19



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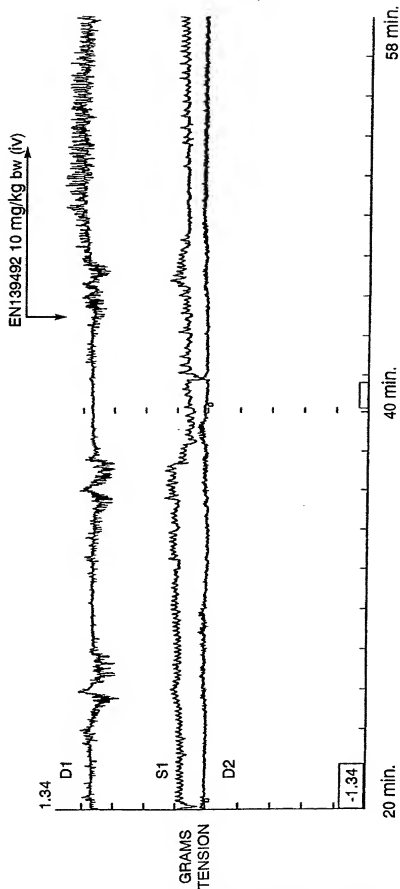


FIG. 20

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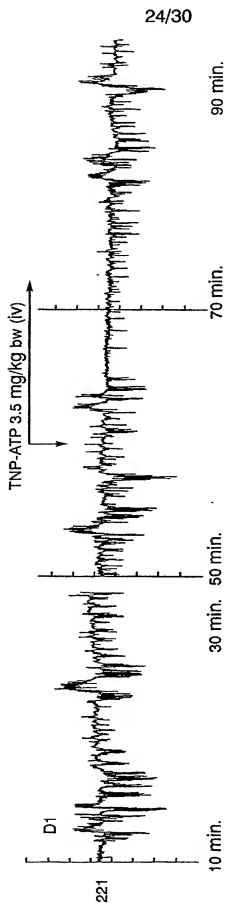


FIG. 21

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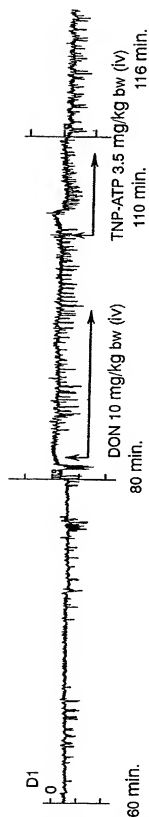


FIG. 22

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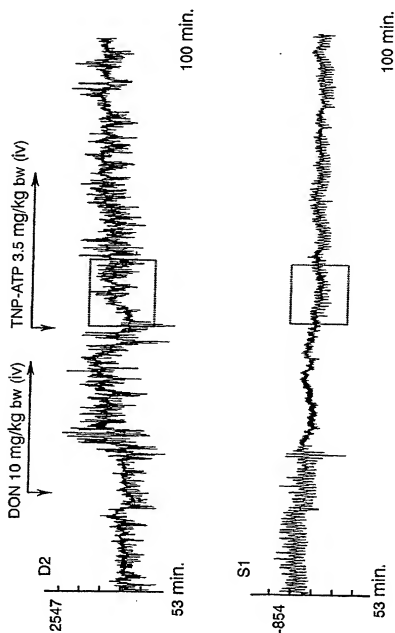


FIG. 23

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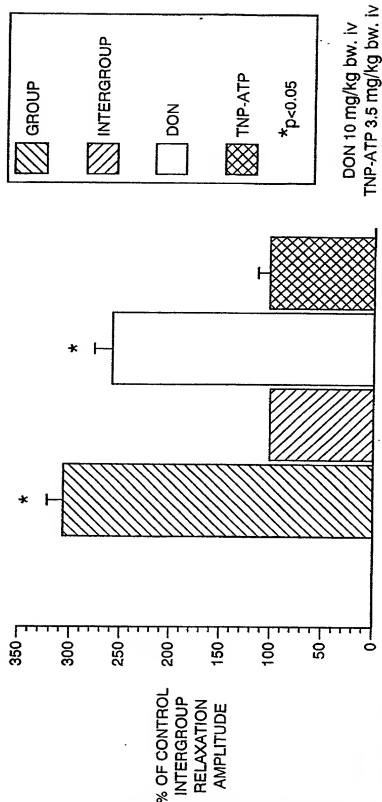


FIG. 24

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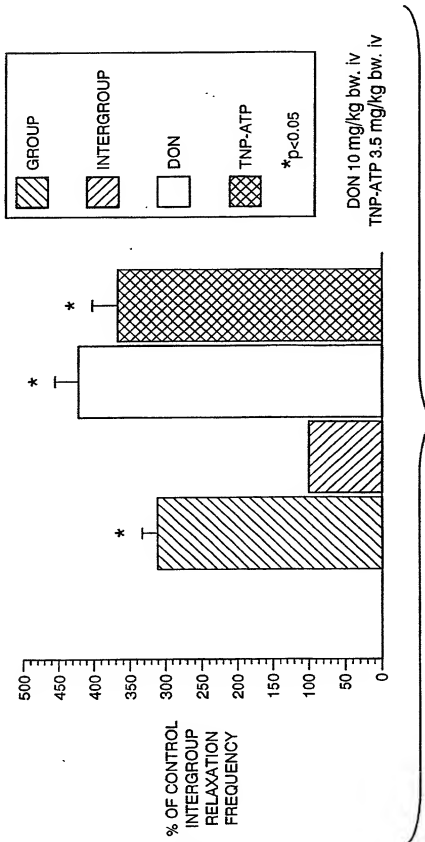


FIG. 25

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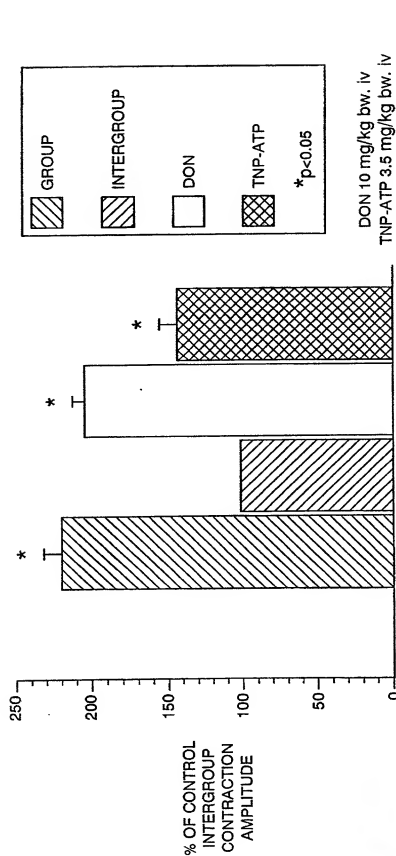


FIG. 26

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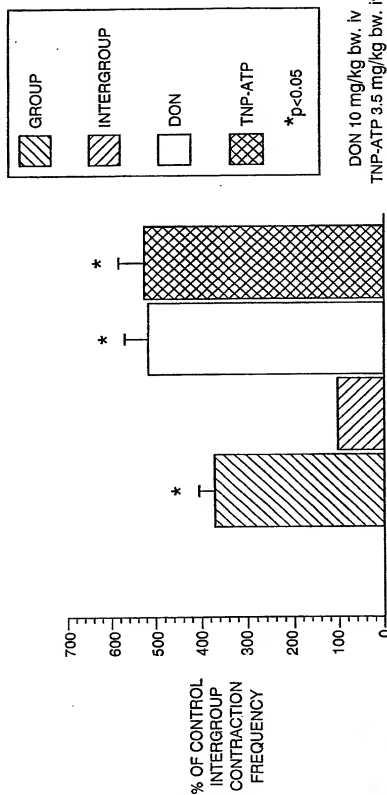


FIG. 27



# COMBINED DECLARATION FOR A PATENT APPLICATION AND POWER OF ATTORNEY

As the below-named inventors, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

## METHODS AND COMPOSITIONS FOR REGULATING GUT MOTILITY AND FOOD INTAKE

the specification of which:

(check one) ☒ [X] is attached hereto.  
☐ [ ] was filed as U.S. application Ser. No. \_\_\_\_\_ on \_\_\_\_\_  
and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I do not know and do not believe that the invention was ever patented or described in any printed publication in any country before my invention thereof or more than one year prior to this application.

I do not know and do not believe that the invention was in public use or on sale in the United States of America more than one year prior to this application.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate filed by me on the same subject having a filing date before that of the application on which priority is claimed:

NONE

(Number)

(Country)

(Day/Month/Year filed)

Priority  
Claimed  
[ ] [ ]  
Yes No

2010102669792-010403

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

<u>60/143,054</u>	<u>6 July 1999</u>	<u>expired</u>
(Application Serial No.)	(Filing Date)	(Status: pending/expired)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States Patent application(s), or under § 365(c) of any PCT international applications designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or international application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

<u>PCT/CA00/00709</u>	<u>6 July 2000</u>	<u>pending</u>
(Application Serial No.)	(Filing Date)	(Status: patented/pending/abandoned)

I hereby appoint	Leon R. Yankwich	Registration No. 30,237
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	David G. O'Brien	Registration No. 46,125
	Regina S. Bautista	Registration No. 46,280

and the firm of Yankwich & Associates, 130 Bishop Allen Drive, Cambridge, MA 02139 as my attorneys and agent, with full authority to prosecute this application and to transact all business before the U.S. Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made in the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, §1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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First inventor's signature

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